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Functional analysis of the clathrin assembly protein, AP180, in *Dictyostelium discoideum*

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Functional analysis of the clathrin assembly protein, AP180, in *Dictyostelium discoideum*

by

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Dedication

To my Mum, Dad and Brother

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Functional analysis of the clathrin assembly protein, AP180, in *Dictyostelium discoideum*

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AP180, an important coat component of clathrin-coated vesicles, is known to assemble clathrin triskelia into cages of uniform size. To gain insight into the relationship between AP180 and clathrin, the gene that encodes AP180 in *Dictyostelium discoideum* was cloned and a mutant strain carrying a deletion in this gene was constructed using homologous recombination. Unlike clathrin mutants, AP180 null cells displayed normal pinocytosis, cytokinesis and development into fruiting bodies. However, AP180 mutant cells were osmosensitive, a phenotype also exhibited by clathrin light chain and clathrin heavy chain mutants. The contractile vacuole in AP180 mutant cells became abnormally large in a hypotonic environment and the cycle of expansion and discharge of the vacuole took twice as long compared to that of wild-type cells. Expression of GFP tagged AP180 showed that it localized to punctae at the plasma membrane, cytoplasm and perinuclear area and that it associated extensively with clathrin at these sites. AP180 also localized to the contractile vacuole and in the absence of AP180 more contractile vacuoles were labeled with clathrin. The association of AP180 with the contractile vacuole was affected

in the absence of clathrin light chain and the internalization of AP180 into cytoplasmic punctae required the presence of clathrin heavy chain.

This work also investigated the dynamics between clathrin, AP180 and AP-2, which is another clathrin assembly protein. A double mutant strain was constructed that had the genes for both AP180 and AP-2 deleted. Of all the clathrin mediated processes examined only osmoregulation was more severe than in the AP180 or AP-2 single mutant cells. The osmosensitivity of the double mutant was an indication that clathrin events at the plasma membrane and the contractile vacuole are linked to some extent. In the absence of both adaptor proteins, the membrane association of clathrin was decreased but not completely abolished whereas the presence of clathrin on the contractile vacuole was markedly decreased. These results present a functional relationship between clathrin, AP180 and AP-2 and suggest that endocytic events mediated by clathrin, AP180 and AP-2 are important in the normal function of the contractile vacuole.

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Chapter 1: Introduction

1.1 CLINICAL IMPLICATIONS OF CLATHRIN MEDIATED ENDOCYTOSIS

Clathrin-mediated endocytosis has received considerable attention from numerous labs over the past years because it is one of the main pathways for the internalization of important receptors, nutrients, and the downregulation of receptors (Brodsky *et al.*, 2001). Clathrin-independent, endocytosis also exists in cells and this pathway has received increased interest since the discovery of the lipid rafts (Nichols and Lippincott-Schwartz, 2001). While this pathway too is both fascinating and crucial in understanding the complex mechanisms of receptor endocytosis and sorting events, its review is beyond the scope of this writing which will focus mostly on the current knowledge about clathrin-mediated endocytosis.

It is not surprising that clathrin mediated endocytosis has important implications in human disease. A classical example is hypercholesterolemia where the low density lipoprotein (LDL) receptor fails to be endocytosed via clathrin mediated endocytosis, leading to excess LDL in the blood (Anderson *et al.*, 1977). The importance of clathrin mediated endocytosis is also underscored in disorders of the nervous system such as Down syndrome, Alzheimer's and Huntington's disease (Velier *et al.*, 1998; Cataldo *et al.*, 2000; Arai *et al.*, 2002). In the case of Down syndrome, the expression of synaptojanin, an important modulator of clathrin-mediated, synaptic vesicle, endocytosis, is much higher than normal, which could affect signaling deficiencies in the developing Down syndrome brain (Arai *et al.*, 2002). In Alzheimer's disease, the endocytic pathway plays a crucial part in the function of proteins, such as the amyloid beta peptide, known to be important determinants of the disease. Altered endocytic pathways that affect the generation of the amyloid beta peptide are thought to link endocytosis and neuronal

changes that occur in sporadic Alzheimer's disease. Also, a decreased expression of two major clathrin adaptor proteins AP180 and AP-2, in patients with Alzheimer's has provided further evidence for the importance of clathrin-mediated endocytic events (Yao *et al.*, 1999; Yao *et al.*, 2000). In Huntington's disease, the cytoplasmic protein Huntingtin has a mutated N-terminal region that causes an abnormal protein interaction, which leads to the pathogenesis of Huntington's disease (Velier *et al.*, 1998). Huntingtin interacts with endocytosed vesicles and proteins such as Huntingtin-interacting protein 1-related (Hip1R), a component of clathrin coated vesicles (Engqvist-Goldstein *et al.*, 2001). Clathrin mediated endocytosis is also exploited by some viruses, such as the influenza virus, to gain entry into cells. In the case of influenza virus, its entry into endosomes and subsequently into acidic compartments facilitates the fusion of the viral envelop with the membrane of the acidic compartment and the release of viral RNA into the cytoplasm. Through clathrin mediated endocytosis, CD4 receptors on the plasma membrane of T helper cells of the immune system are internalized because of the action of an HIV protein named Nef. The internalization of CD4 on T helper cells interferes with the normal function of T helper cells and this gives an opportunity for the HIV virus to spread. Moreover, clathrin mediated endocytosis has been linked to cancer. One recent example came from patients with lymphoid myeloid leukemia. In these patients a chromosomal translocation that results in the fusion between a clathrin assembly protein, AP180 or CALM, and a putative transcription factor, AF10, is thought to be a key determinant for developing leukemia (Dreyling *et al.*, 1996).

1.2 CLATHRIN STRUCTURE AND FUNCTION

1.2.1 Discovery of clathrin

Before the discovery of clathrin 42 years ago, the concept of endocytosis emerged from the observations of the Russian Nobel prize winner Ilya Mechnikov almost 100 years ago. Mechnikov noticed that certain cells engulfed litmus particles that turned from blue to red indicating the presence of an acidic compartment in the cell. He named this process Phagocytosis (from the Greek meaning eating cells) and the concept of uptake and dynamic trafficking has fascinated many ever since (Klemparskaya, 1983). In 1964, Thomas Roth and Keith Porter provided the first evidence of the existence of clathrin coated pits following studies on the uptake of yolk proteins in the oocyte of the mosquito *Aedes aegypti* (Roth and Porter, 1964). Using electron microscopy they showed the formation of "bristle" coats on invaginated oocyte cell membrane and the pinching off from the membrane into the oocyte as "bristle-coated vesicles" (Roth and Porter, 1964) (Figure 1.1). Several labs thereafter provided further evidence for the existence of coated vesicles in other organisms (Friend and Farquhar, 1967; Palade and Bruns, 1968; Kanaseki and Kadota, 1969; Gray and Willis, 1970). About a decade after the initial observations of coated vesicles, a study from Heuser and Reese established the importance of coated vesicles in the recycling of synaptic membrane following stimulation of frog muscle cells (Heuser and Reese, 1973). Shortly thereafter Pearse purified and biochemically characterized clathrin from pigs brain (Pearse, 1975). She also found that the coated vesicles were 600 Å in diameter and that the coat contained essentially one single protein of molecular weight 190 kDa that she named clathrin. Pearse further noted that the size of the vesicles was the same as those from brain synaptosomes described in an earlier study (Kanaseki and Kadota, 1969) Since then, and

with the remarkable advances in microscopy, there has been a major additional progress in understanding clathrin structure and function.

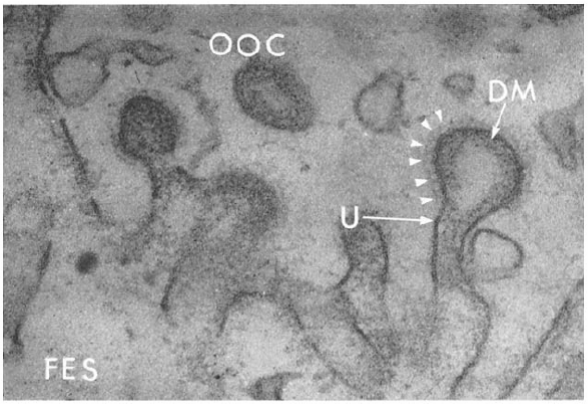


Figure 1.1: EM of clathrin "bristles". Electron image from Roth and Porter, 1964 showing a dense material (DM) on the outside of the oocyte (OOC) membrane that is endocytosed and the bristles that surround the invaginated membrane (arrows). FES, follicular epithelium; U, unit membrane (Roth and Porter, 1964).

1.2.2 Clathrin structure and assembly

The first images of clathrin structures came from rotary shadowing electron microscopy from several groups working on different organisms (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981; Payne and Schekman, 1985; Riddelle-Spencer and O'Halloran, 1997). These images showed that clathrin forms a triskelion (three legged) structure made up of three identical clathrin heavy chains (CHC) (Kirchhausen *et al.*, 1986) (Figure 1.2).

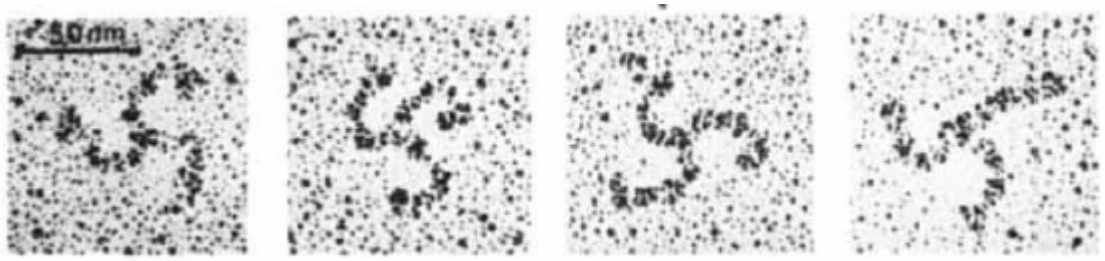


Figure 1.2: Electron micrograph of clathrin triskelions following rotary shadowing. Three clathrin heavy chains connected at their carboxyl terminals make up the central hub (Brodsky *et al.*, 1987).

CHC is a ~190 kDa protein that is made up of three distinct regions, the proximal, the distal and the terminal region (Kirchhausen, 2000). The carboxyl terminal of each CHC connects to each other to form the central hub of the triskelion. This area serves as a CHC trimerization site and as a binding site for three identical clathrin light chains (CLC) (Ungewickell and Branton, 1981)(Figure 1.3). One interesting structural feature of the proximal and distal region is the presence of a 145-residue motif that is repeated seven times. The function of these repeats is not well understood but it is speculated that they mediate protein-protein interactions (Smith and Pearse, 1999). A crystal structure of the terminal domain showed that it is a seven-blade -propeller similar to that formed by WD repeats found in heterotrimeric G-proteins (Wall *et al.*, 1995; Lambright *et al.*, 1996; Sondek *et al.*, 1996). The “blades” of the -propeller serve as binding sites for various clathrin assembly and accessory proteins of the endocytic pathway (ter Haar *et al.*, 1998).

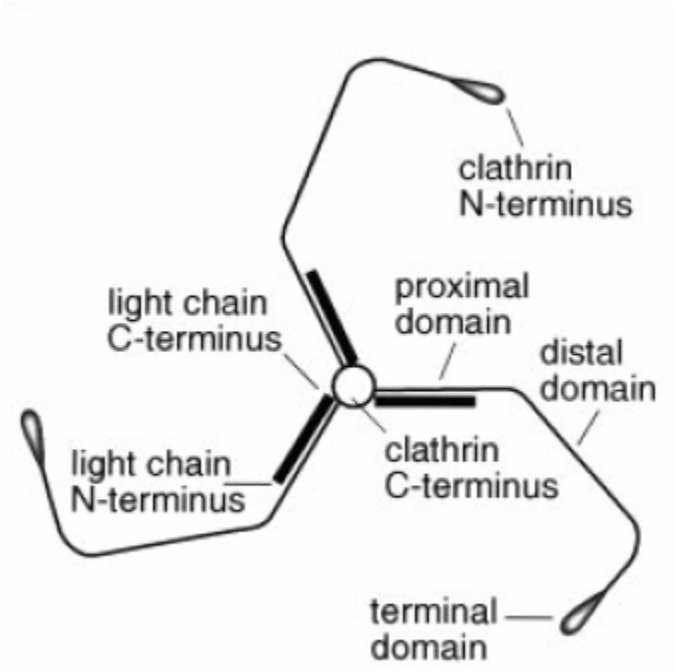


Figure 1.3: Organization of a clathrin triskelion. Each heavy chain binds to one light chain at the central hub (clathrin C-terminus region). The heavy chains have three distinct function regions; the proximal, distal and terminal regions (Smith *et al.*, 1998)

Clathrin light chains bind noncovalently together to the heavy chain, and are thought to be involved in clathrin assembly and function (Brodsky *et al.*, 1987; Brodsky *et al.*, 1991; Wang *et al.*, 2003). The triskelions of CHC can self assemble and the absence of CLC under certain buffer conditions does not interfere with the inherent assembly properties of the triskelia (Ungewickell and Ungewickell, 1991; Liu *et al.*, 1995). However, in vivo studies in yeast and the soil amoeba *Dictyostelium discoideum* pointed out the importance of CLC in the trimerization of CHC (Huang *et al.*, 1997) and the overall stability of the structure (Huang *et al.*, 1997; Wang *et al.*, 2003). How this

remarkable structure comes together to form a clathrin cage and what factors affect this assembly has been the focus of many research groups. Initially, the structure of cages assembled from clathrin triskelions was solved to 21Å using cryo-electron microscopy (Smith *et al.*, 1998; Heldwein *et al.*, 2004; Cheng *et al.*, 2006; Fotin *et al.*, 2006). The map showed that the three triskelions and the three light chains interact through the proximal and distal domains and that the legs intertwine to form regular hexagonal and pentagonal lattices (Smith *et al.*, 1998; Fotin *et al.*, 2006). This in vitro structure complements electron micrographs of deep-etched surfaces of unroofed cells that have captured clathrin assembly into lattices and coated pits on the cytoplasmic face of the plasma membrane where clathrin mediated endocytosis occurs (Figure 1.4)(Heuser, 1980) . This brings us to the topic of clathrin assembly at the plasma membrane and the factors that contribute to the assembly of clathrin triskelia into pentagonal or hexagonal coated pits.

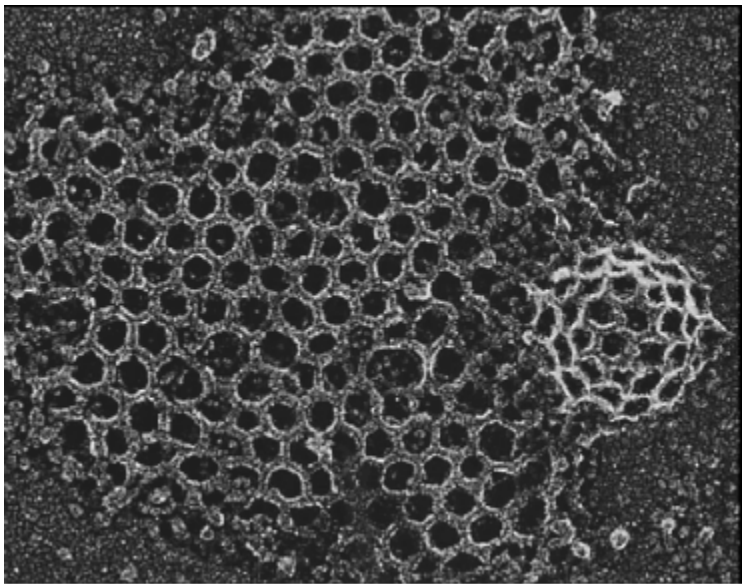


Figure 1.4: Clathrin triskelia assemble into flat lattices and coated pits. Deep-etch image from fixed mammalian tissue culture cells showing the assembly of clathrin triskelia into a hexagonal and pentagonal arrangement as a flat lattice in contact with the membrane and as a mature coated pit ready to pinch off from the membrane (Heuser, 1980).

1.3 CLATHRIN-MEDIATED ENDOCYTOSIS AND MEMBRANE TRAFFIC

1.3.1 Overview

The endocytic pathway is a complex cellular pathway where proteins are internalized into vesicles and sorted to their destinations. The initial stages of endocytosis occur at the membrane and depend on the presence of clathrin and its efficient assembly. Once clathrin triskelia form a coat around the invaginated membrane, the vesicle pinches off from the membrane and is carried into the cytoplasm. The clathrin coat disassembles from the vesicle and now the "naked" vesicle can be delivered to and fuse with other membrane-limited cytoplasmic sorting compartments such as endosomes (Figure 1.5). Over the years numerous endocytic accessory proteins and key regulators in this pathway have been unveiled. For example, the assembly of clathrin at the plasma membrane is driven by assembly proteins such as AP-2 and AP180 (Lindner and Ungewickell, 1992; Hao *et al.*, 1999).

Tethering of clathrin to the plasma membrane also involves AP-2 and AP180, as well as the endocytic protein epsin (Kirchhausen, 1999; Wendland *et al.*, 1999). Accessory proteins such as Hip1R (Huntingtin-interaction protein 1-related) and Eps15 also facilitate tethering of clathrin to the plasma membrane by their association with the actin cytoskeleton and protein interactions with other endocytic proteins (Benmerah *et al.*, 1996; van Delft *et al.*, 1997; Wendland and Emr, 1998; Bennett *et al.*, 2001;

Engqvist-Goldstein *et al.*, 2001; Confalonieri and Di Fiore, 2002). The scission of clathrin coated pits from the membrane is driven by dynamin and actin (Robinson, 1994; Brodin *et al.*, 2000) and finally the uncoating of the vesicle and the release of clathrin triskelia back into the cytoplasm is mediated by auxillin and the heat shock protein Hsc70 (Holstein *et al.*, 1996; Barouch *et al.*, 1997). While many more proteins are involved in these events, this review will highlight the function of clathrin assembly proteins.

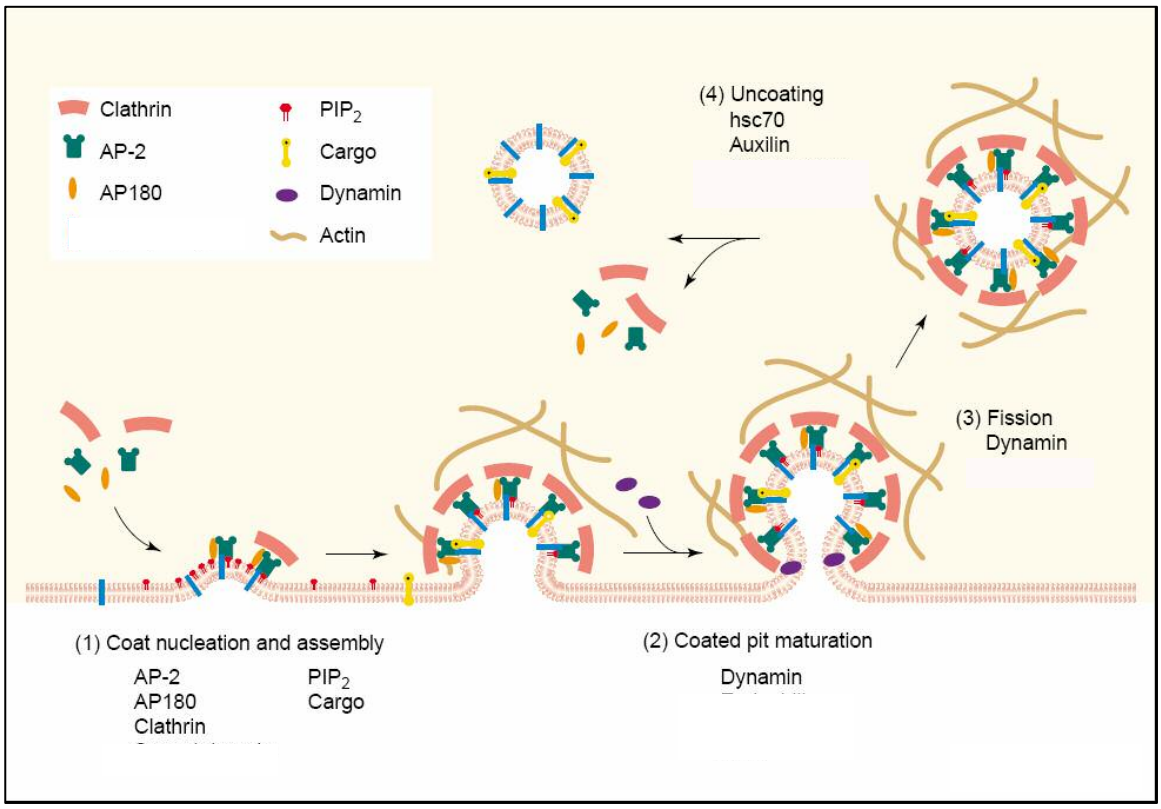


Figure 1.5: Overview of clathrin-mediated endocytosis. Clathrin coat initiation occurs at the membrane through the activity of assembly proteins such as AP-2 and AP180. The target cargo is invaginated into a maturing pit that severs from the plasma membrane through the action of dynamin. Internalization of the coated pit is followed by the

uncoating of clathrin from the vesicle mediated by Auxillin and hsc70 (Takei and Haucke, 2001).

1.3.2 The role of adaptor proteins

Second only to clathrin in abundance, the adaptor proteins (AP's) are major components of clathrin-coated vesicles. There are four heterotetrameric adaptor proteins: AP-1, which functions at the Trans Golgi Network (TGN), AP-2 which is associated with the plasma membrane, AP-3 which is involved in protein sorting form the TGN to endosomal-lysosomal compartments, and AP-4 which associates with the trans-Golgi network and is thought to play a role in the sorting of integral membrane proteins (Dell'Angelica *et al.*, 1998; Le Borgne and Hoflack, 1998; Dell'Angelica *et al.*, 1999). Their structure is similar, consisting of two large subunits (, , or for AP-2, AP-1, AP-3 and AP-4 respectively and) of about 100 kDa, a medium size subunit of about 50 kDa called the μ subunit and a small subunit of about 20 kDa. While little is known about the function of AP-3 and AP-4, much progress has been made regarding the structure and function of AP-2 and AP-1. These adaptor proteins bind directly to clathrin and in vitro studies have shown that AP-2 assembles clathrin triskelia into cages (Zaremba and Keen, 1983; Pearse and Robinson, 1984; Ahle and Ungewickell, 1989). The subunit of the adaptors contains a sequence known as the clathrin box (Dell'Angelica *et al.*, 1998) that confers binding to the terminal domain of the clathrin triskelion (Gallusser and Kirchhausen, 1993; Shih *et al.*, 1995). Furthermore, the subunit of AP-2 has been shown to bind polyphosphoinositides such as PtdIns(4,5)P_2 on the plasma membrane (Gaidarov *et al.*, 1996). Based on these properties, the adaptor proteins are thought to recruit clathrin to the membrane and initiate the assembly of a clathrin coat. However, further characterization of the adaptor subunits revealed that the μ subunit is involved in

the recognition of membrane cargo proteins through tyrosine-type sorting signals found in the cargo (Owen and Evans, 1998; Bonifacino and Traub, 2003). Therefore, the function of the heterotetrameric adaptor proteins also appears to be in specifying the site of clathrin assembly through the recognition of desired cargo at the membrane.

In addition to the tetrameric AP's, monomeric adaptors for clathrin on the plasma membrane have also recently been identified. For example, -arrestins link specific cargo to the clathrin scaffold. -arrestins bind to activated 2-adrenergic receptors which are seven-transmembrane G-coupled proteins, and this binding results in their efficient removal from the cell surface via clathrin-coated vesicles (Ferguson *et al.*, 1996; Goodman *et al.*, 1996; Zhang *et al.*, 1996; Lin *et al.*, 1998). -arrestin 1 and -arrestin 2 contain the conserved clathrin binding box at their carboxyl terminus and have been shown to bind directly to clathrin (Krupnick *et al.*, 1997) whereas their amino-terminus confers binding to inositol phosphates including PtdIns-4,5-P_2 (Gaidarov and Keen, 1999). Therefore, arrestins provide another link between the internalization of cell membrane cargo and clathrin coat assembly. Epsin, another monomeric clathrin adaptor, binds clathrin and PtdIns-4,5-P_2 on the plasma membrane and drives curvature of the membrane during the early stages of invagination (Ford *et al.*, 2002; Wendland, 2002; Stahelin *et al.*, 2003). Epsin also contains ubiquitin interacting motifs (UIMs) which are important for receptor internalization at the plasma membrane (Oldham *et al.*, 2002; Shih *et al.*, 2002). Another adaptor protein that links clathrin to the plasma membrane through PtdIns-4,5-P_2 interaction is Hip1R. Hip1R also binds actin and the yeast homologue, Sla2p, organizes F-actin and plays an important role in endocytosis (Bennett *et al.*, 2001; Engqvist-Goldstein *et al.*, 2001; Baggett *et al.*, 2003; Chen and Brodsky, 2004; Newpher and Lemmon, 2006). Clathrin assembly is facilitated by the monomeric adaptor protein

AP180 which has potent clathrin assembly activity and tethers clathrin to the plasma membrane via lipid interaction.

1.3.3 The role of AP180

AP180 was first purified from coated vesicles of bovine brain (Ahle and Ungewickell, 1986) and it is the third most abundant protein in neuronal endocytic vesicles besides clathrin and AP2. Studies from several groups have shown that AP180 localizes with clathrin coated synaptic vesicles at the nerve terminal plasma membrane where it interacts with AP2 and phosphatidylinositol-4,5-bisphosphate (PIP_2)(Norris *et al.*, 1995; Ye *et al.*, 1995; Hao *et al.*, 1997). Furthermore, AP180 binds directly to clathrin triskelia and promotes their assembly into cages of homogeneous size *in vitro* (Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988; Ye and Lafer, 1995a). Homologues of the mammalian AP180 have been found in *Drosophila, Caenorhabditis elegan*s and squid. The *Drosophila* homologue, *LAP* (**L**ike **AP**180) colocalizes with clathrin at synaptic boutons and plays a role in synaptic vesicle endocytosis (Zhang *et al.*, 1998). Interestingly, *LAP* mutants show abnormal clathrin localization at the nerve terminals, decreased endocytosis and an increase in the diameter of synaptic vesicles (Zhang *et al.*, 1998). The latter observation suggested that AP180 could be acting as a regulator of vesicle size through reassembly of clathrin cages. The homologue of AP180 in *C. elegans*, *Unc-11*, has recently been shown to be required for the efficient localization of synaptobrevin to the synaptic sites (Nonet *et al.*, 1999). As seen in LAP mutants, vesicle diameter is significantly larger in *Unc-11* mutant cells than in wild-type cells (Nonet *et al.*, 1999). However homozygous *unc-11* mutants are viable and capable of forming synaptic vesicles during endocytosis suggesting that *Unc-11* is not essential for clathrin mediated endocytosis.

Until recently AP180 was thought to function exclusively in neurons. However, homologues of AP180 have been identified in yeast, humans and now in *Dictyostelium*. In the yeast *S. cerevisiae* there are two genes that code for AP180 related proteins, Yap180a and Yap180b, which are 43% identical to one another (Wendland and Emr, 1998). Surprisingly, strains that lack both Yap180a and Yap180b remain viable, grow even at high temperatures and, most importantly, show no defects in endocytosis (Wendland and Emr, 1998). In fact, studies using yeast strains that lack all clathrin adaptor proteins, including Yap180a and Yap180b, have shown that clathrin function and recruitment to the membrane is not dependent on these adaptor /assembly proteins, at least in yeast (Huang *et al.*, 1999). Interestingly, Pan1p, an endocytic protein and an activator of the Arp2/3 complex in yeast, was found to bind to Yap180a, thereby providing evidence of an important link between clathrin binding proteins and the actin cytoskeleton (Duncan *et al.*, 2001).

Recent studies of the non-neuronal mammalian homologue of AP180, also called CALM (**C**lathrin **A**ssembly **L**ymphoid **M**yeloid leukemia gene), have found that overexpression of AP180 in mammalian cells affects both the localization of clathrin and endocytosis (Tebar *et al.*, 1999). AP180/CALM was first identified from a lymphoma cell line in which a chromosomal translocation resulted in a gene fusion between AF10 (a putative transcription factor) and CALM (Dreyling *et al.*, 1996). Sequence analysis of CALM showed that it shared homology with the neuronal AP180, providing evidence for the first time of a translocation that involved a putative clathrin binding protein (Dreyling *et al.*, 1996). Subsequently, Tebar and colleagues provided strong evidence through a series of experiments, that CALM is an important component of clathrin coated vesicles. First they showed that CALM binds directly to clathrin using GST pull down and immunoprecipitation assays. This result was supported by immunofluorescence staining

of HeLA cells that showed significant colocalization between the two proteins. Second, they found that overexpression of CALM inhibited the endocytosis of the transferrin receptor and epidermal growth factor receptor, which are internalized by the clathrin-mediated pathway (Tebar *et al.*, 1999). Moreover, Tebar et al showed that CALM localizes to the cell periphery as well as the TGN and that overexpression of GFP-CALM resulted in the dispersion of clathrin from the TGN. This loss of clathrin from the TGN was assessed using the mannose-6-phosphate receptor (M6PR) which uses the clathrin-mediated pathway to move from the TGN to endosomes. In cells overexpressing CALM, the sorting of M6PR from TGN to endosomes was inhibited. Taken together, these findings suggest that CALM is involved in the two major clathrin-mediated pathways: clathrin-mediated endocytosis from the plasma membrane and clathrin-mediated sorting at the TGN (Tebar *et al.*, 1999). However, the exact role of CALM in either process still remains elusive.

1.3.2 Clathrin at the Golgi

Clathrin mediated endocytosis is not restricted to the plasma membrane. Protein internalization via clathrin coated pits can occur from any membranous organelle such as the trans Golgi network (TGN). Indeed, immunofluorescence images localize clathrin to the TGN and disruption of TGN using brefeldin A disperses clathrin from that area (Kent *et al.*, 2002). Furthermore, clathrin colocalizes with AP-1, which is the adaptor exclusively found at the Golgi. Clathrin is necessary for the correct sorting of the cation-independed mannose 6-phosphate receptor and carboxypeptidase D, which localize to the trans Golgi network (Kornfeld and Mellman, 1989; Harasaki *et al.*, 2005), and in yeast, clathrin is required for maintaining the localization of proteins such as Kex2p (a processing endopeptidase) at the TGN (Chu *et al.*, 1999). Monomeric adaptor proteins that have been localized to the Golgi network include the GGAs [Golgi-localized, -ear-

containing and Arf (ADP-ribosylation factor)-binding proteins]. These bind to and recruit clathrin to the TGN (Puertollano *et al.*, 2001). GGAs mediate the sorting of mannose 6-phosphate receptors between the trans-Golgi and endosomes. Also in yeast, GGAs are involved in the sorting of vacuolar hydrolase precursors and trafficking of SNAREs from the Golgi to the prevacuolar compartments (Bonifacino, 2004). Therefore, the importance of clathrin-mediated endocytosis is highlighted not only at the plasma membrane but also at the TGN.

1.4 THE ROLE OF CLATHRIN IN *DICTYOSTELIUM DISCOIDEUM*

1.4.1 *Dictyostelium* as a model system

Dictyostelium discoideum is a social amoeba that lives in the soil and feeds on bacteria. In the vegetative stage, *Dictyostelium* cells divide every 8-10 hours by cell fission. When nutrients are depleted, a developmental cycle is triggered. The steps of the developmental cycle are well defined, and one can visualize the whole process using a simple dissecting microscope. When *Dictyostelium* cells starve, cAMP is secreted to signal neighboring cells to start chemotaxis (Parent and Devreotes, 1999; Firtel and Chung, 2000). A community of about 100000 cells migrates towards an aggregation center to form a slug that crawls in search of light. The cells that make up the slug then differentiate into one of two types: stalk cells or spore cells. Subsequently, a fruiting body forms, composed of a stalk and a sorus (spore head). This process takes about 24 hours to complete and all stages of the developmental cycle can be reproduced in the laboratory. When nutrients become available again, the dormancy ends and the spores germinate into amoeboid cells. The life cycle of *Dictyostelium* is ideal to study fundamental developmental processes such as cell polarity, which *Dictyostelium* cells adopt as they

move towards the aggregation center, chemotaxis, cell migration (which resembles neutrophil migration), and cell differentiation. *Dictyostelium* has six chromosomes (Cox *et al.*, 1990). Its 34 Mb genome is intermediate in size between yeast and *C. elegans* and it contains an estimated 10000 genes (Loomis *et al.*, 1995). The haploid genome allows easy genetic manipulations and the construction of strains with entire genes deleted using homologous recombination. One drawback of the *Dictyostelium* genome is the high A/T content (even in coding regions) that poses some difficulty when carrying out molecular genetics. The introduction of plasmids into *Dictyostelium* cells is carried out by electroporation and subsequent selection with a selectable marker. Unfortunately, only two reliable selectable markers for use in *Dictyostelium* cells are in standard use, although recently a group has successfully used the *Cre-loxP* system to delete multiples genes using only one selectable marker (Faix *et al.*, 2004).

Dictyostelium is also suited for morphological studies. The 10 micron size of *Dictyostelium* cells allows the visualization of dynamic processes such as phagocytosis, cell division, chemotaxis and endocytic events as well as organelle morphology. With an array of fluorescent tags and markers available, cellular processes such as the endocytic pathway can now be studied in real time. The endocytic pathway in *Dictyostelium* has received significant attention because it is essential to the cells, it is highly dynamic, it closely resembles that of higher eukaryotes and it is easily tracked using microscopy.

1.4.1 Clathrin in *Dictyostelium discoideum*

Dictyostelium discoideum has one gene for the clathrin heavy chain (CHC) and one gene for the clathrin light chain (CLC) (O'Halloran and Anderson, 1992a; Wang *et al.*, 2003). *Dictyostelium* CHC is a 1,694 amino acid, 194 kDa protein that shares 57% amino acid identity with the mammalian CHC (O'Halloran and Anderson, 1992a). The structure of clathrin triskelia in *Dictyostelium* resembles that of mammalian and yeast

clathrin as seen by rotary shadowing (Riddelle-Spencer and O'Halloran, 1997). Clathrin coated vesicles have been seen on unroofed cells using electron microscopy (Heuser, 2006) and thin section electron microscopy revealed the presence of approximately 340 clathrin coated vesicles in each cell of average vesicle diameter of 93 nm (Swanson *et al.*, 1981). The importance of CHC in *Dictyostelium* was evident from the initial studies done by O'Halloran and Anderson when they knocked down the gene expression for CHC using anti-sense RNA (O'Halloran and Anderson, 1992a). CHC mutants displayed severe defects in endocytosis, growth rates, pinocytosis, osmoregulation, cell migration rates and development (O'Halloran and Anderson, 1992b). Remarkably, these cells remained viable and showed no obvious deficiencies in phagocytosis. Further characterization of a complete CHC null mutant revealed a role of clathrin in early development and the differentiation of spore cells but not stalk cells during late stages of the developmental cycle (Niswonger and O'Halloran, 1997a). Interestingly, CHC null cells were found to be deficient in cytokinesis. Normally *Dictyostelium* cells can grow and divide in suspension cultures. However, CHC null cells fail to do so and become multinucleated (Niswonger and O'Halloran, 1997b). These results supported the idea that membrane trafficking events are important during cell division and cytokinesis. In fact, myosin II, which normally localizes to the contractile ring during cell division, fails to assemble at that site in the absence of CHC (Niswonger and O'Halloran, 1997b). *Dictyostelium* cells also require CHC for the sorting of lysosomal enzymes (Ruscetti *et al.*, 1994). Normally, 95% of -mannosidase is sorted from the Golgi to the endosomes and finally to the lysosomes during which it is processed to its mature form, whereas the remaining 5% is rapidly excreted through the default secretory pathway (Cardelli *et al.*, 1986; Richardson *et al.*, 1988; Cardelli *et al.*, 1990) . In CHC mutants about 30% of the lysosomal enzyme precursor of -mannosidase was missorted to the default secretory pathway instead of to

the endosomal/lysosomal pathway. (Ruscetti *et al.*, 1994). Furthermore, -mannosidase as well as -glucosidase failed to be secreted normally as they displayed increased intracellular activity compared to wild-type cells (Ruscetti *et al.*, 1994).

Clathrin light chain (CLC) and its role in clathrin-mediated processes has also been studied in *Dictyostelium* (Wang *et al.*, 2003). CLC mutant cells displayed the same deficiencies as CHC null cells such as in development, cytokinesis and osmoregulation (Wang *et al.*, 2003). Furthermore, the absence of CLC caused a decrease in the association of CHC with the plasma membrane pointing to a role of CLC in the efficient self assembly of clathrin triskelions (Ungewickell and Ungewickell, 1991; Liu *et al.*, 1995; Wang *et al.*, 2003). In addition, structure-function analysis of the *Dictyostelium* CLC revealed that the conserved carboxyl terminal part of the protein is both necessary and sufficient for its function (Wang *et al.*, 2006). Collectively, these results show that the role of clathrin in *Dictyostelium* is multifaceted and one that closely resembles clathrin in higher eukaryotes.

1.5 THE CONTRACTILE VACUOLE

1.5.1 Overview

Dictyostelium cells are naturally found in the soil where they are often exposed to water. Therefore *Dictyostelium* cells require a robust and efficient system to cope with the changes in osmolarity surrounding them. This task is carried out by a specialized organelle known as the contractile vacuole. The contractile vacuole is a bipartite organelle composed of tubules and bladders or cisternae that interconnect to make a reticular contractile vacuole network. This organelle is highly dynamic and is set in motion as soon as the osmotic environment changes. As the hypo-osmotic media, such as

water, enters the cell, the tubules collect the excess water and feed it to a main bladder that expands as it draws in more water. As the bladder expands and the tubules shorten, the bladder moves closer to the plasma membrane. When it reaches its maximum capacity the bladder fuses briefly with the membrane and expels its contents to the extracellular milieu (Gerisch *et al.*, 2002). Following the collapse of the contractile vacuole bladder against the plasma membrane, the tubules elongate as they continue to collect water and the main bladder regenerates to resume the cycle. This dynamic process appears choreographed and is carried out faithfully with much the same pattern every round of expansion and expulsion. This indicates that it is a highly controlled process brought about by an array of different proteins. The mechanism of contractile vacuole activity and the proteins involved in the regulation and function of the contractile vacuole are subjects that have received much interest albeit more important questions still remain unanswered.

1.5.2 Structure of the contractile vacuole in *Paramecium* and *Dictyostelium*

The first recording for the existence of a contractile vacuole in protozoa dates back to the 1700s by Lazzaro Spallazani (Doetsch, 1976; Allen, 2000). A significant amount of information about the function of the contractile vacuole has come from studies carried out not only in *Dictyostelium* but also from a close relative the ciliate protozoan *Paramecium*. *Paramecium* cells are bigger than amoeba and contain an organized contractile vacuole system that consists of two contractile vacuoles, one on the posterior and one on the anterior part of the cell (Figure 1.6). The contractile vacuole in *Paramecium* is made up of collecting canals that collect water from the cell cytoplasm, the ampullae which deliver the water from the collecting canals to the main contractile vacuole, and the contractile vacuole which accumulates the water and expels it to the outside of the cell. The collecting canals are supported by microtubules that run across

the contractile vacuole system. The collecting canals also associate with the decorated spongiome which contains V-H^+ATPase proton pumps that can be visualized with an antibody against the V1 portion of the V-H+ATPase (Wassmer *et al.*, 2005). *Paramecium* contractile vacuoles function similarly to *Dictyostelium* contractile vacuoles but with important differences. In *Paramecium* the collecting canals draw in water from the cytoplasm and deliver it to the ampullae (small membrane bulges) which directly feed into the main contractile vacuole. As the contractile vacuole collects water and expands the pore in the membrane is closed and the ampullae remain in contact with the main vacuole. When the contractile vacuole reaches its maximum size and rounds up into a sphere, the ampullae break away from the contractile vacuole as the permanent pore opens and the contractile vacuole expels its contents. The detached ampullae that continue to draw in water from the canals fuse back with the main contractile vacuole and the cycle starts again.

Figure 1.6: The contractile vacuole in *Paramecium*. The ciliate *Paramecium* contains two contractile vacuoles on each end of the cell. It is made up of smooth spongiomes (the main contractile vacuole bladder, the ampullae and the collecting canals and decorated spongiomes (collecting canals). This cell is labeled with a monoclonal antibody against

the V-H+ATPase proton pump that is found only on the decorated spongiome on the collecting canals (Ishida *et al.*, 1996).

In *Dictyostelium* cells the contractile vacuole system does not conform to the same strict organization of canals, ampullae and bladders. Instead the contractile vacuole is a reticular network of tubules and cisternae that interconvert and function dynamically throughout the cell. An important difference between the contractile vacuole system in *Paramyceium* and *Dictyostelium* is that *Dictyostelium* lacks a permanent pore in the membrane. Instead, the discharging contractile vacuole is able to fuse at any site of the membrane. Despite the brief fusion between the contractile vacuole and the plasma membrane the two entities never mix (Gabriel *et al.*, 1999). Another major difference is that *Dictyostelium* does not have a defined number of contractile vacuoles. The numbers and activity of the contractile vacuoles fluctuate in each cell depending on the osmotic changes of the environment. Normally hypotonic media increases the numbers and activity of the contractile vacuole. Heuser has provided remarkable images of the contractile vacuole in *Dictyostelium* using transmission electron microscopy of unroofed cells and showed clearly that the contractile vacuole network is a meshwork of tubules and bladders that interconnect to form one unified system (Heuser *et al.*, 1993)(Figure 1.7). The common feature between the contractile vacuoles in these two model organisms is the tubules and bladders. In both organisms these function similarly to collect water in the tubules or canals, deliver the water to the main contractile vacuole, discharge water when bladder collapses and regenerate to start the cycle over again (Figure 1.8).

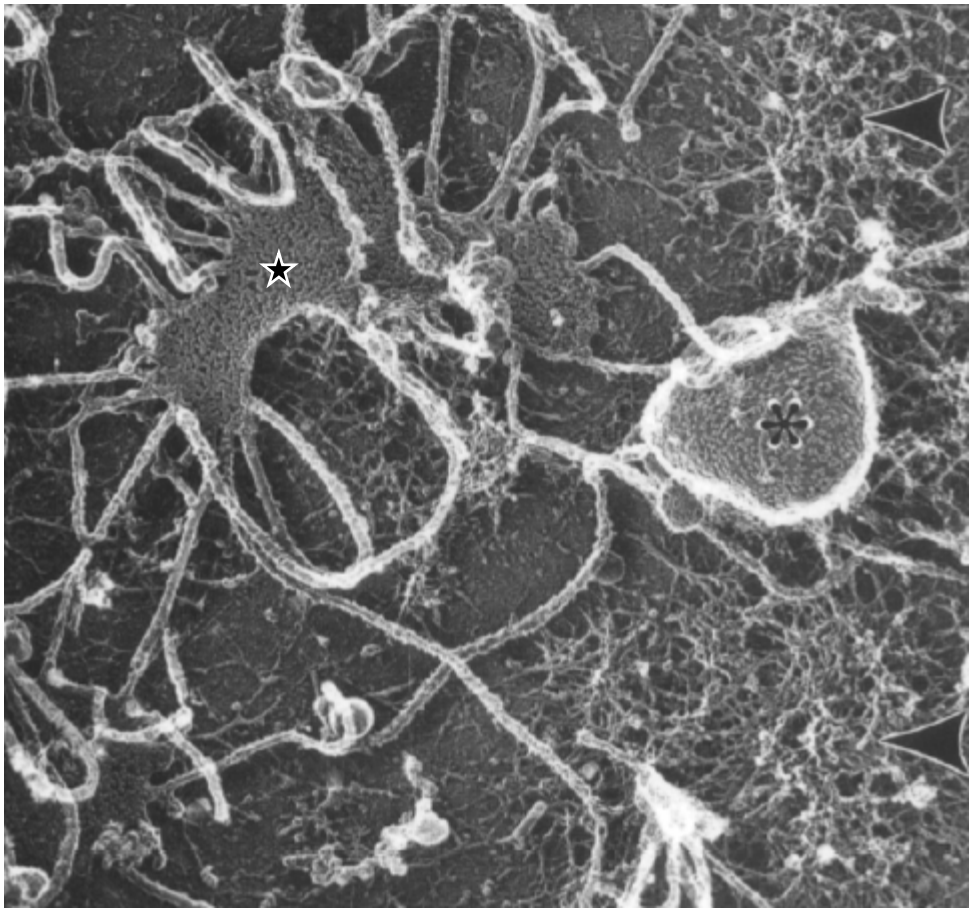


Figure 1.7: The *Dictyostelium discoideum* contractile vacuole. Transmission electron microscopy (TEM) image of an unroofed *Dictyostelium* cell showing an extensive network of tubules that feed into bladders. Two bladders are shown in this image, one is partially filled (asterisk) and the other one is flat (star). Also visible are cytoskeletal structures on the plasma membrane (arrowheads)(Heuser *et al.*, 1993).

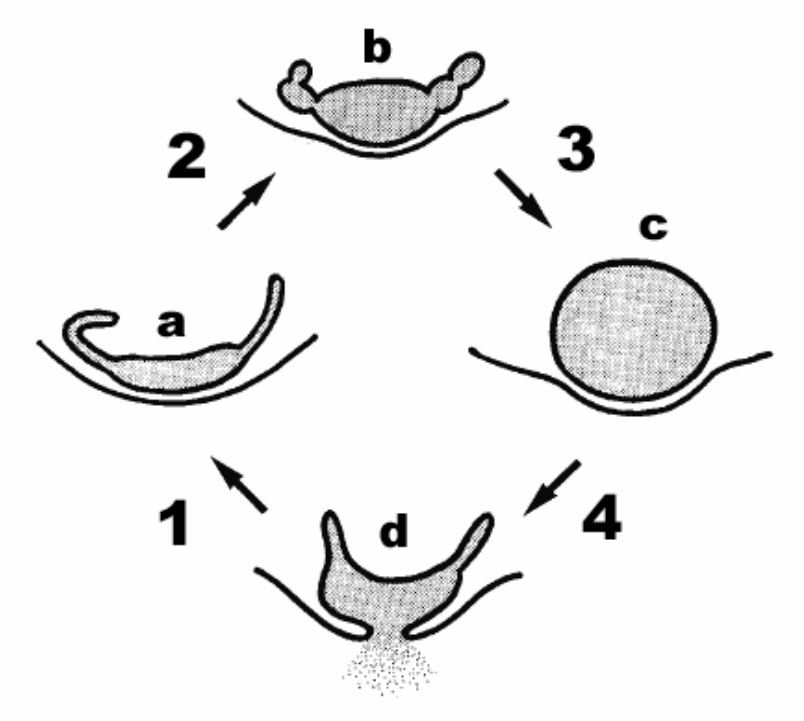


Figure 1.8: Proposed cycle of the contractile vacuole in *Dictyostelium*. Tubules that are connected to the main bladder rush water in from the intracellular space (a). As the amount of water increases the volume of the bladder also increases (b) until it becomes fully expanded and round (c). The tubules shorten into the main bladder that moves close to the plasma membrane. A temporary pore opens and the fusion of the contractile vacuole with the membrane results in the release of fluid and the collapse of the bladder (d). This is followed by tubulation of the contractile vacuole and the initiation of another cycle (Heuser *et al.*, 1993).

1.4.3 Contractile vacuole resident proteins and their function

The contractile vacuole of *Dictyostelium* is believed to be derived from the Golgi as evident from the presence of O-glycosylated proteins on the membrane of the

contractile vacuole (Gabriel *et al.*, 1999). It is distinct from the endocytic pathway and endocytic compartments. When cells expressing Dajumin-GFP (Green Fluorescent Protein), a marker specific for the contractile vacuole, were fed fluorescent dextran to label endosomes, there was no overlap between the endocytic marker and the contractile vacuole (Gabriel *et al.*, 1999). However three proteins, RabD, golvesin and the vacuolar H^+-ATPase (V-H+ATPase) proton pump, are found on both endocytic compartments and the contractile vacuole (Bush *et al.*, 1994; Temesvari *et al.*, 1996; Schneider *et al.*, 2000). The vacuolar proton pump is important for contractile vacuole function and is believed to drive the exchange of water from the cytosol across the contractile vacuole membrane. Little is known about the contents of the contractile vacuole other than it is a hypotonic fluid and that it is not acidic despite the presence of the proton pumps on the contractile vacuole membrane. How water enters the contractile vacuole lumen remains unclear, but it has been proposed that the proton pumps create a positive electrical charge on the inside of the contractile vacuole, causing first anions, and then water to travel down their concentration gradient into the contractile vacuole. This must occur without upsetting the ion balance of the cell. A possible candidate for this exchange is bicarbonate ions that are by-products of respiration (Heuser *et al.*, 1993). The contractile vacuole is also believed to regulate intracellular Ca^{+2} concentrations. Several lines of evidence support this idea. First, antibodies against the Ca^{+2} binding protein calmodulin stain the contractile vacuole (Zhu and Clarke, 1992) .Second, a P-type Ca^{+2} ATPase, named PAT1, was cloned in *Dictyostelium* and was shown to localize to the contractile vacuole (Moniakis *et al.*, 1995; Moniakis *et al.*, 1999). Finally, purified contractile vacuoles were shown to store and release Ca^{+2} and mutants with a disorganized contractile vacuole system exhibit decreased Ca^{+2} vesicular transport to the contractile vacuole (Gerald *et al.*, 2002; Malchow *et al.*, 2006).

1.4.4 *Dictyostelium* mutants that affect the contractile vacuole

Several groups have cloned and characterized proteins that localize to and function at the contractile vacuole. The deletion of the genes encoding these proteins affects the function of the contractile vacuole in different ways indicating differences in their contributions to contractile vacuole activity. One such protein is Drainin, so named because mutant cells that lack this protein display enormous contractile vacuoles that fail to drain (Becker *et al.*, 1999). Drainin is a peripheral membrane protein that associates with the contractile vacuole throughout the cycle of expansion and expulsion. This protein has homologues in yeast, *C. elegans* and humans and belongs to a subgroup of GTP-ase activating proteins. Therefore it is thought that drainin is involved in a volume-sensing signaling pathway that allows the timely fusion of the vacuole to the plasma membrane (Becker *et al.*, 1999). Another interesting protein that resides on the contractile vacuole is LvsA (Large Volume Sphere) (Gerald *et al.*, 2002). GFP-tagged LvsA colocalizes with calmodulin and the V-H^+ATPase pump on the contractile vacuole and deletion of this gene causes a disorganization of the contractile vacuole and the dissociation of calmodulin from this compartment (Gerald *et al.*, 2002). Interestingly, LvsA only localizes to the contractile vacuole when it is ready to expel and remains associated until the final stages of expulsion. After this discharge, LvsA dissociates from the contractile vacuole membrane (Gerald *et al.*, 2002). The transient timing of LvsA association with the contractile vacuole suggests the existence of a cellular mechanism that senses when the contractile vacuole reaches its maximum capacity and that sets in motion the subsequent discharge and collapse of the vacuolar membrane. In drainin mutant cells, LvsA fails to disengage from the abnormally large and defective contractile vacuoles, which suggests that LvsA acts before drainin and does not require drainin to associate with the contractile vacuole (Wu *et al.*, 2004).

While the contractile vacuole system and the endocytic compartments are separate entities, recent studies have shown that proteins involved in clathrin-mediated endocytosis are also involved in the biogenesis and activity of the contractile vacuole system. One such protein is the Golgi clathrin adaptor protein AP-1. AP-1 does not localize to the contractile vacuole but associates with the Golgi apparatus from which the contractile vacuole is believed to be derived (Lefkir *et al.*, 2003). Most importantly, *Dictyostelium* cells with a deletion of the medium subunit of AP-1 (apm1$^-$), exhibit severe osmosensitivity, no contractile vacuole network, and several mislocalized contractile vacuole resident proteins (Lefkir *et al.*, 2003). Clathrin itself plays a vital role in contractile vacuole activity and this has been a topic that remains largely unexplored yet extremely intriguing. Clathrin heavy chain and clathrin light chain mutant cells have osmoregulation deficiencies. As mentioned above, clathrin heavy chain in *Dictyostelium* is involved in many processes including cytokinesis, development, pinocytosis, sorting and secretion of lysosomal enzymes, and cell movement (O'Halloran and Anderson, 1992b, 1992a; Ruscetti *et al.*, 1994; Niswonger and O'Halloran, 1997b, 1997a; Riddelle-Spencer and O'Halloran, 1997; Damer and O'Halloran, 2000; Gerald *et al.*, 2001). One of the interesting phenotypes of clathrin heavy chain null cells is their extreme osmosensitivity and the absence of a functional contractile vacuole (O'Halloran and Anderson, 1992b). The presence of clathrin at the contractile vacuole has been somewhat of a mystery because EM images of *Dictyostelium* contractile vacuoles rarely show the presence of clathrin triskelia and when they are found there they usually decorate the ends of the tubules (Heuser, personal communication). Under physiological conditions and normal contractile vacuole activity, it is thought that clathrin is not involved in the recycling of contractile vacuole membrane following its collapse on the plasma membrane (Heuser, 2006), although this is still a subject under investigation. I have used

fluorescence microscopy to confirm the dynamic association of clathrin with the contractile vacuole and possible functions for clathrin on this organelle will be discussed in more detail in following chapters. As mentioned above, RabD is another endocytic protein that functions in phagocytosis as well as in the regulation of contractile vacuole activity (Harris *et al.*, 2001; Harris and Cardelli, 2002). Dominant negative RabD in *Dictyostelium* display an extensive reticular contractile vacuole network and abnormal sized contractile vacuole bladder possibly due to defects in membrane trafficking events (Harris *et al.*, 2001).

Proteins of the endocytic pathway may directly influence contraction of the contractile bladder. Quick-freeze deep-etch EM micrographs of *Dictyostelium* cells show that the contractile vacuole network is devoid of any major cytoskeletal components such as actin and myosin (Heuser, 2006) therefore contraction of the bladder is likely not driven by the movement of actomyosin filaments. Instead, the contraction of the bladder could result from endocytic proteins that bind the membrane and rearrange the local phospholipid composition so that tubulation occurs and the bladder collapses. Given that the nature of contractile vacuole dynamics requires multiple events of membrane deformation, tubulation and rearrangement, endocytic proteins are good candidates for playing important roles for contractile vacuole function. These proteins could couple the rearrangement of local lipids, clathrin binding, protein trafficking and sorting events to the dynamics of the contractile vacuole, and thus contribute to the generation and function of the contractile vacuole.

Chapter 2: The monomeric clathrin assembly protein, AP180, regulates contractile vacuole size in *Dictyostelium discoideum*

2.1 INTRODUCTION

Endocytosis via clathrin coated vesicles is an essential process for the regulated uptake of nutrients and the trafficking of membrane receptors. While clathrin is the principal structural player in building a coated vesicle, numerous adaptor and accessory proteins fine-tune and regulate endocytosis. (Lafer, 2002). Together they assemble clathrin triskelia onto membranes to construct a clathrin coated pit (Kirchhausen, 2000; Brodsky *et al.*, 2001). Four heterotetrameric adaptor proteins have been identified; each is thought to function at a different cellular location (Kirchhausen, 1999). At the plasma membrane the heterotetrameric protein AP-2 recruits clathrin and initiates the assembly of clathrin triskelia into lattices (Gallusser and Kirchhausen, 1993; Owen *et al.*, 2000). Recently, monomeric assembly proteins have been identified at the plasma membrane that could similarly regulate clathrin traffic. The monomeric assembly protein, AP180, was first purified from coated vesicles of bovine brain (Ahle and Ungewickell, 1986), although a non-neuronal homologue of AP180, CALM, was identified more recently (Dreyling *et al.*, 1996; Tebar *et al.*, 1999). AP180 binds directly to clathrin and promotes assembly of clathrin triskelia into cages of uniform size (Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988; Benmerah *et al.*, 1995).

Analysis of mutants of the AP180 orthologs in *D. melanogaster* and *C. elegans* shows that AP180 regulates synaptic vesicle size as well as the sorting of synaptic proteins such as synaptobrevin (Zhang *et al.*, 1998; Nonet *et al.*, 1999; Bao *et al.*, 2005). In mammalian cells, reduction of AP180 results in irregular clathrin lattices, demonstrating an important role for AP180 in the assembly of clathrin into geometrically

precise coated vesicles (Meyerholz *et al.*, 2005). Furthermore, recent studies show that AP180 is involved in the internalization of some receptors like the EGF receptor, but not of others such as the transferrin receptor (Huang *et al.*, 2004). In yeast, the role of AP180 in clathrin-mediated endocytosis is less clear. Deletion of both genes encoding the AP180 orthologs (yAP180a and yAP180b) show no defects in any clathrin mediated processes (Huang *et al.*, 1999).

I*n vitro* experiments show that AP180 binds clathrin and phosphoinositides such as PIP2 and promotes clathrin assembly on lipid monolayers (Ford *et al.*, 2001). Binding of AP180 to PIP2 is mediated by an NH_2-terminal homology domain called the ANTH (**A**P180 **N**-**t**erminal **h**omology) domain that is conserved in all members of the AP180 family (Norris *et al.*, 1995; Ye *et al.*, 1995; Hao *et al.*, 1997; Ford *et al.*, 2001; Mao *et al.*, 2001). Other endocytic proteins such as epsin have at their amino terminus a structurally similar ENTH domain (**E**psin **N**-**t**erminal **h**omology). Interestingly, binding of ENTH-domain-containing proteins such as epsin, to PIP2 induces curvature of a lipid monolayer whereas AP180 fails to do so (Ford *et al.*, 2002; Stahelin *et al.*, 2003). This points to important mechanistic differences between various protein components of the endocytic machinery.

Here we examined the intracellular role of AP180 in the social amoeba, *Dictyostelium discoideum*. While *Dictyostelium* AP180 colocalized with clathrin on the plasma membrane of wild-type cells, AP180 null cells displayed a normal distribution of clathrin on the plasma membrane. However AP180 knockouts were deficient in osmoregulation mediated by the contractile vacuole, a process where clathrin is also a key regulator. Collectively our results suggest that AP180 is a clathrin assembly protein with unique contributions to the regulation of contractile vacuole size.

2.2 RESULTS

2.2.1 *Dictyostelium* AP180 contains membrane-binding and clathrin adaptor signature motifs

Members of the AP180 family have at their amino-terminus a signature domain called the ANTH (**A**P180 **N**-**t**erminus **H**omology) domain. This domain has been extensively studied and established as a PtdIns(4,5)P2 binding domain (Norris *et al.*, 1995; Ye *et al.*, 1995; Hao *et al.*, 1997). Using the amino acid sequence for the ANTH domain of the mammalian neuronal AP180 protein (Ahle and Ungewickell, 1986), I searched the database of the *Dictyostelium* genome and found a single gene that showed high homology to this sequence. Analysis of the retrieved sequence showed a conserved lysine-rich motif ^{28}*KATx6PKxKH* at the amino-terminus which agreed with the ANTH domain consensus sequence *(K/G)A(T/I)x6(P/L/V)KxK(H/Y)* (Kay *et al.*, 1999; Ford *et al.*, 2001). The first 300 amino acids at the amino-terminus shared significant homology with the ANTH domain of the AP180 orthologs from *D. melanogaster* (Lap;32.3%) (Zhang *et al.*, 1998); *C. elegans* (UNC-11;28.7%) (Nonet *et al.*, 1999); *H. sapiens* (CALM ;32.7%) (Tebar *et al.*, 1999); the neuronal isoform from *Bos taurus* (AP180 ;34%) (Ahle and Ungewickell, 1986; Kohtz and Puszkin, 1988); and *S. cerevisae* (YAP180; 20%) (Wendland and Emr, 1998). Analysis of the amino acid sequence carboxyl-terminus to the ANTH domain revealed a clathrin box, LINFD (amino acids 360-364) (Morgan *et al*., 2000), closely followed by an AP-2 binding motif, DPF (amino acids 407-409) (Hao *et al.*, 1999; Owen *et al.*, 1999; Traub *et al.*, 1999; Owen and Luzio, 2000; Owen *et al.*, 2000) and an NPF motif (amino acids 459-461) which confers binding to EH-domain-containing proteins such as Eps15 (Benmerah *et al.*, 1998; Cupers *et al.*, 1998; de Beer *et al.*, 1998; Lohi *et al.*, 1998; McPherson *et al.*, 1998; Roos and Kelly, 1998; Wendland and Emr, 1998; Whitehead *et al.*, 1998; Yamabhai *et al.*, 1998; Salcini

et al., 1999) (Figure 2.1). Because of the amino acid sequence homology and the presence of all signature motifs common to AP180 family members, including the non-neuronal mammalian AP180 ortholog, CALM, we named this gene *clmA*. *ClmA* encoded a protein predicted to contain 695 amino acids with a molecular mass of 79.7 kDa. A BLAST search of the *Dictyostelium* genome database located *clmA* to chromosome 3.

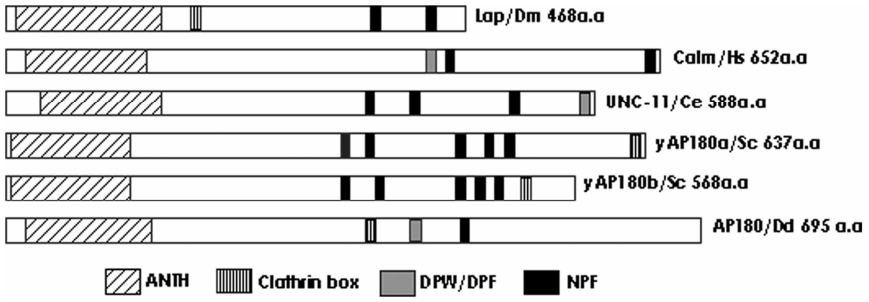


Figure 2.1: *Dictyostelium* AP180 belongs to the AP180 family**.** All members of the AP180 family have an amino-terminal ANTH signature domain that confers binding to phospholipids at the plasma membrane. Most family members also contain the amino acid sequence DLL or L(L/I)(D/E/N)(L/F)(D/E), a short motif that binds to clathrin; a DPW/DPF or FXDXF motif that confers binding to the major adaptor protein AP-2, and NPF/W, a motif that binds to EH domain-containing proteins. Dm: *D. melanogaster*; Hs: *H. sapiens*; Ce: *C. elegans*; Sc: *S. cerevisiae*; Dd: *D. discoideum*.

2.2.2 AP180 colocalizes with clathrin on the plasma membrane

As monomeric adaptors for clathrin, members of the AP180 family associate with clathrin at the plasma membrane, and the clathrin box in the *Dictyostelium* AP180 sequence suggested that it might also associate with clathrin. To examine the intracellular

location of the *Dictyostelium* ortholog, I made a plasmid that expressed the AP180 protein tagged with Green Fluorescent Protein (GFP). Growing *Dictyostelium* cells that expressed GFP-AP180 were gently flattened, fixed and stained with an antibody against clathrin light chain followed by a secondary antibody conjugated to Texas Red (Figure 2.2A). GFP-AP180 localized as discrete punctae at the plasma membrane and in the cytoplasm. In addition, GFP-AP180 frequently labeled the nucleus, and colocalized with the nuclear stain 4'-6-diamidino-2-phenylindole (DAPI) (data not shown). Labeling cells with an antibody against clathrin showed that AP180 and clathrin colocalized in many punctae on the plasma membrane and in the cytoplasm. However AP180 punctae that lacked clathrin were also found on the plasma membrane. Clathrin punctae that lacked GFP-AP180 were more frequently observed in the cytoplasm whereas the majority of clathrin punctae on the plasma membrane contained AP180.

2.2.3 AP180 associates with the contractile vacuole

Close examination of fixed or living amoebae cells expressing GFP-AP180 revealed that the tagged protein localized as punctae not only at the plasma membrane and within the cytoplasm, but also to ring-like structures. These structures were identified as contractile vacuoles when visualized under Differential Interference Contrast (DIC) in living cells because they expanded and contracted periodically (Heuser *et al.*, 1993). In addition to the vacuoles, GFP-AP180 also decorated the tubules of the contractile network that fed into the main vacuole (Figure 2.2B). Costaining fixed cells with an anti-clathrin antibody revealed that these vacuoles contained punctae of clathrin and of GFP-AP180 (Figure 2.2C).

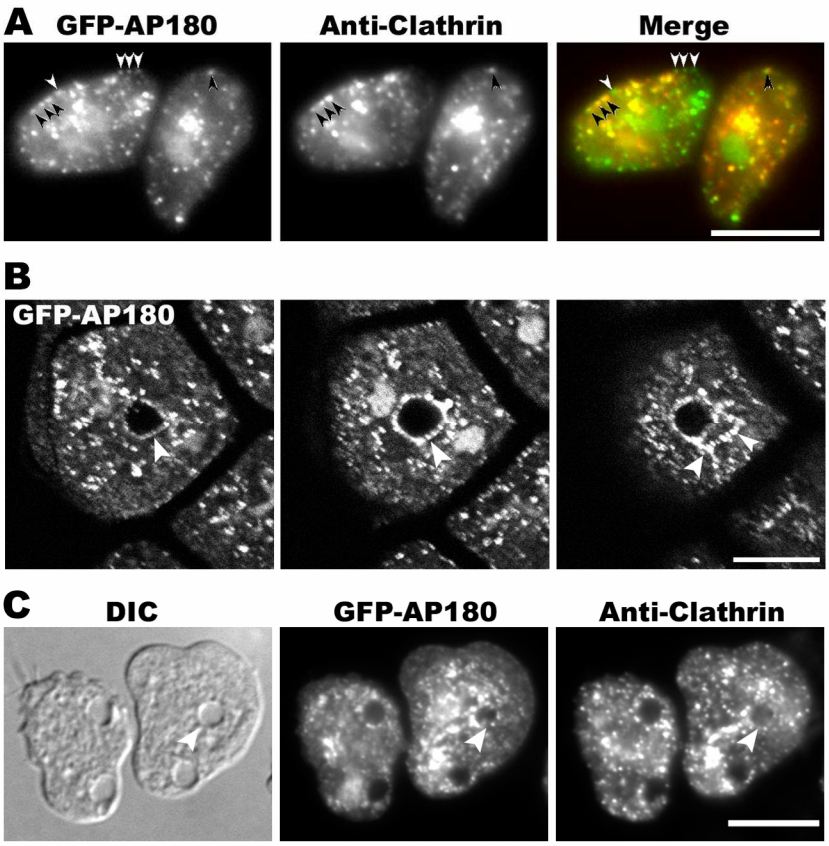


Figure 2.2: Localization of clathrin and AP180. **A**. AP180 and clathrin colocalize. Cells expressing GFP-AP180 were fixed and stained with an anti-clathrin light chain antibody detected with a Texas Red conjugated secondary antibody. The majority of AP180 punctae at the plasma membrane colocalized with clathrin (black arrows) although some AP180 punctae lacked clathrin (white arrows). Merge shows GFP-AP180 in green and clathrin in red. Scale bar, 10 microns. **B**. Z-series of cells expressing GFP-AP180 fixed and imaged by confocal microscopy. GFP-AP180 punctae decorated the contractile vacuole bladder (arrow, left and middle panels) as well as the tubules radiating from it (arrows, right panel). The two bright signals adjacent to the central bladder represent two

nuclei. Each panel is 0.8 microns apart. Scale bar, 10 microns. **C**. Cells expressing GFP-AP180 were fixed and stained with an anti-clathrin light chain antibody followed by Texas Red conjugated secondary antibody. Punctae of GFP-AP180 and clathrin outlined the contractile vacuole (arrow). Scale bar, 10 microns.

2.2.4 Clathrin mutants display an altered distribution of AP180

To test whether clathrin was required for the clustering of AP180 into punctae on the plasma membrane, we expressed GFP-tagged AP180 in cells that lacked either the clathrin heavy chain gene or the clathrin light chain gene (Ruscetti *et al*, 1994 and Wang *et al*, 2003) (Figure 2.3A). In clathrin heavy chain null cells, the GFP-AP180 punctae at the plasma membrane remained visible however most of the cytoplasmic punctae were lost. In general, punctae on the membrane of clathrin heavy chain null cells were not as bright and were more diffuse than those of wild-type cells. In most clathrin light chain null cells, the GFP-AP180 remained associated with punctae on the plasma membrane and scattered throughout the cytoplasm, a distribution similar to wild-type cells. In about 20% of cells that lacked clathrin light chain, GFP-AP180 accumulated on one side of the plasma membrane, a pattern never seen in wild-type cells (Figure 2.3A, inset).

In addition, clathrin light chain null cells had a pronounced difference in the association of AP180 with the contractile vacuole. Examination of clathrin light chain null cells revealed contractile vacuoles that were occasionally labeled with GFP-tagged AP180. However this association was strikingly less frequent than in wild-type cells expressing GFP-tagged AP180. Whereas 65% of the contractile vacuoles in wild-type cells were labeled with AP180, only 25% of contractile vacuoles in clathrin light chain null cells were labeled with AP180 (Figure 2.3B).

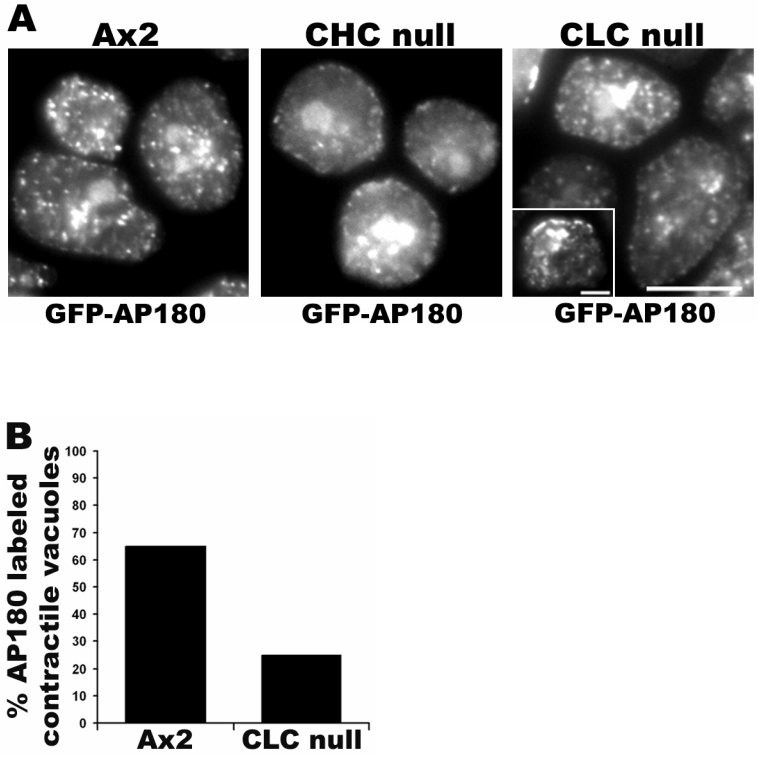


Figure 2.3: Localization of GFP-AP180. A. Wild-type cells (Ax2), clathrin heavy chain null cells (CHC null) and clathrin light chain null cells (CLC null) expressing GFP-AP180 were fixed and imaged with fluorescence microscopy. In CHC null cells, GFP-AP180 punctae in the cytoplasm were absent. In most CLC null cells, GFP-AP180 retained punctae on the plasma membrane and cytoplasm. In some CLC null cells, GFP-AP180 formed a cap on the plasma membrane (inset). Scale bar, 10 microns. Inset: Scale bar, 5 microns. B. AP180 associates less frequently with the contractile vacuoles of CLC null cells. Wild-type Ax2 cells (n=43) and CLC null cells (n=59) expressing GFP-AP180 were incubated in water, fixed, and scored for AP180 localization at the contractile vacuole.

2.2.5 Clathrin localization on the plasma membrane is not affected in cells that lack AP180

Members of the AP180 family associate with clathrin and assemble clathrin triskelia into cages *in vitro* (Ahle and Ungewickell, 1986; Ye and Lafer, 1995a). To test whether *Dictyostelium* AP180 is required for the association of clathrin with cellular membranes, we constructed AP180 null cells using homologous recombination to replace a portion of the coding sequence of the *clmA* gene with a blasticidin marker. Replacement within the *clmA* gene was confirmed by PCR and the absence of the AP180 protein in AP180 null mutants was verified by western blot analysis (data not shown). To test whether *Dictyostelium* AP180 is required for the association of clathrin with cellular membranes, we assessed the distribution of clathrin in wild-type and AP180 null cells using an antibody against clathrin light chain and immunofluorescence microscopy. As shown previously in wild-type cells, clathrin localized as punctae at the plasma membrane, cytoplasm and perinuclear region (Damer and O'Halloran, 2000). This localization pattern was unchanged in cells that lacked AP180 (Figure 2.4A). To examine directly the association of clathrin with intracellular membranes, we performed differential cell fractionation. In both wild-type cells and AP180 null cells, clathrin fractionated into the high speed (100,000 x g) pellet that contains membranes (Figure 2.4B). These results suggested that clathrin retained its ability to associate with intracellular membranes even in the absence of AP180.

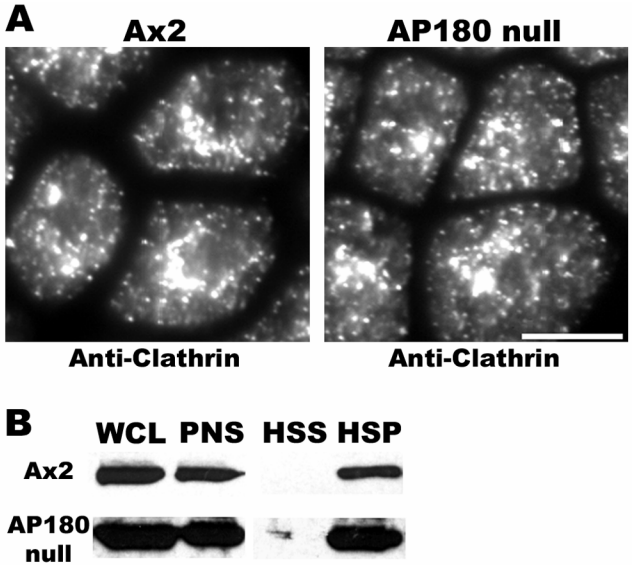


Figure 2.4: Membrane-associated clathrin punctae assemble in the absence of AP180. A. Wild-type cells (Ax2) and AP180 null cells (AP180 null) fixed and stained with an anti-clathrin light chain antibody. Scale bar, 10 microns. B. Differential fractionation of wild-type cells (Ax2) and AP180 mutants (AP180 null) analyzed in immunoblots stained with anti-clathrin heavy chain show similar distribution of clathrin. WCL, whole cell lysate; PNS, Post-Nuclear Supernatant; HSS, High Speed Supernatant and HSP, High Speed Pellet.

2.2.6 Clathrin dynamics at the contractile vacuole are altered in the absence of AP180

Both clathrin light chain and clathrin heavy chain null cells display an osmoregulation defect. To examine the relationship between the contractile vacuole, clathrin and AP180, we utilized a GFP-tagged construct of clathrin light chain and examined its behavior in wild-type cells and AP180 null cells. While the immunofluorescence and fractionation data showed that most clathrin remained

associated with membranes in the absence of AP180, examination of the contractile vacuole revealed an important difference: when wild-type and AP180 null cells expressing GFP-clc were shifted into water, and filmed under low light conditions to maintain contractile vacuole activity, both the wild-type and AP180 null cells had clathrin associated with the contractile vacuole (Figure 2.5A). In such hypotonic environment, cells showed a transient association of clathrin with the bladder and the tubules of the contractile vacuole during all stages of contractile vacuole activity. Clathrin punctae on the contractile vacuole became most evident when the bladder was fully expanded before fusion. These punctae remained visible until the complete discharge of the contractile vacuole. Following the collapse of the bladder into the tubular network, clathrin punctae dispersed into the cytoplasm. This pattern of association of clathrin with the contractile vacuole was also seen in AP180 null cells. However, in the mutants, clathrin punctae at the contractile vacuole were more frequently associated with the contractile vacuole than in wild-type cells. In wild-type cells, about half of the contractile vacuoles were labeled with clathrin (47%, n=19 contractile vacuoles) for at least a portion of the contractile vacuole cycle (Figure 2.5B). Strikingly, AP180 null cells showed more contractile vacuoles labeled with clathrin (90%, n=20) during the contractile vacuole cycle (Figure 2.5B).

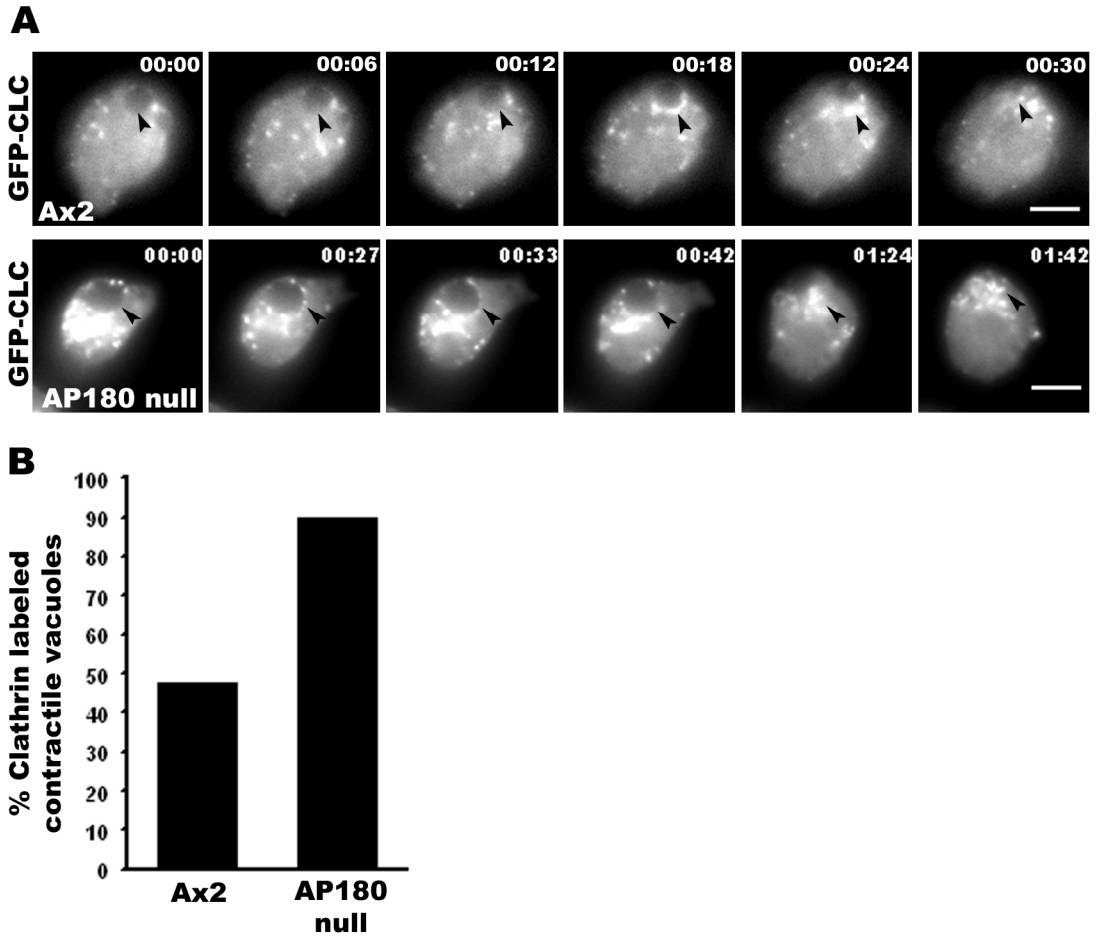


Figure 2.5: The association of clathrin with the contractile vacuole**.** A. Time lapse of living wild-type (Ax2) and AP180 null cells expressing GFP-clc after shifting from media to water. Clathrin localizes to the expanding and discharging contractile vacuole (arrow) in wild-type and AP180 null cells. See accompanying videos, Movie 1 and Movie 2. B. An increased number of contractile vacuoles associate with clathrin in AP180 null cells. Wild-type Ax2 cells (n=19) and AP180 null cells (n=20) expressing GFP-CLC were incubated in water and scored for the presence of clathrin on the contractile vacuole.

2.2.7 AP180 null cells display wild-type endocytosis, development and cytokinesis

To understand how AP180 might contribute to development and cellular function in *Dictyostelium*, I examined the phenotype of AP180 null cells. I assessed these mutant cells for deficiencies associated with *Dictyostelium* clathrin mutants, including fluid-phase endocytosis, development, cytokinesis and osmoregulation (Niswonger and O'Halloran, 1997b, 1997a; Wang *et al.*, 2003). To examine fluid-phase endocytosis, we compared the ability of wild-type cells and AP180 null cells to internalize a fluid-phase marker, FITC-Dextran. I found that AP180 null cells were able to internalize FITC-Dextran as well as wild-type cells (Figure 2.6A). Both clathrin heavy chain and clathrin light chain mutants also display defects in development as shown by their inability to form fruiting bodies (Niswonger and O'Halloran, 1997a; Wang *et al.*, 2003). In contrast, AP180 mutants formed robust fruiting bodies at the same rate as wild-type cells (Figure 2.6B). Furthermore, the sorus of AP180 null cells contained spores that displayed the normal oblong morphology as wild-type cells (Figure 2.6C). AP180 null cells grew well in suspension cultures without forming multinucleated cells, indicating that cytokinesis was also normal in these mutants (data not shown).

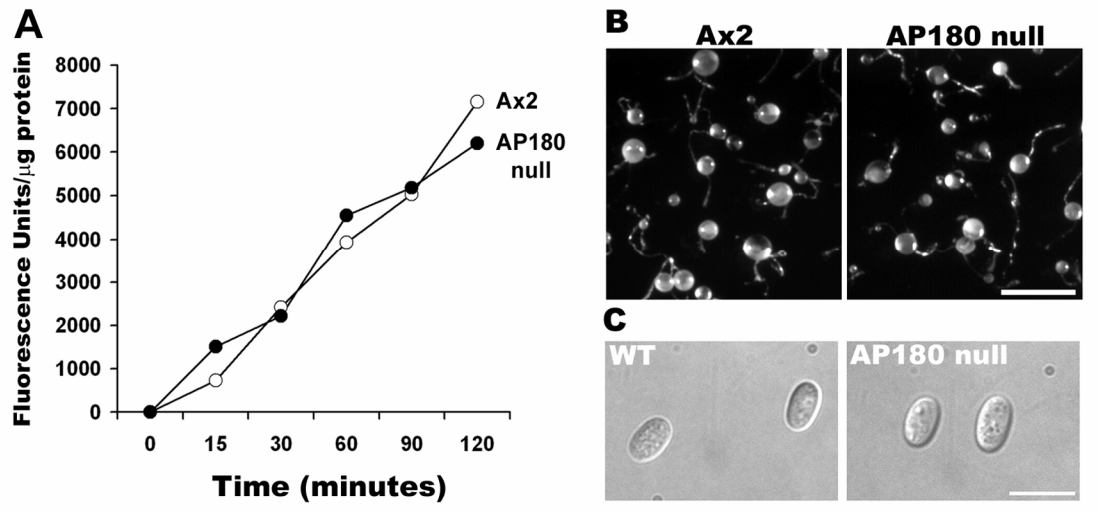
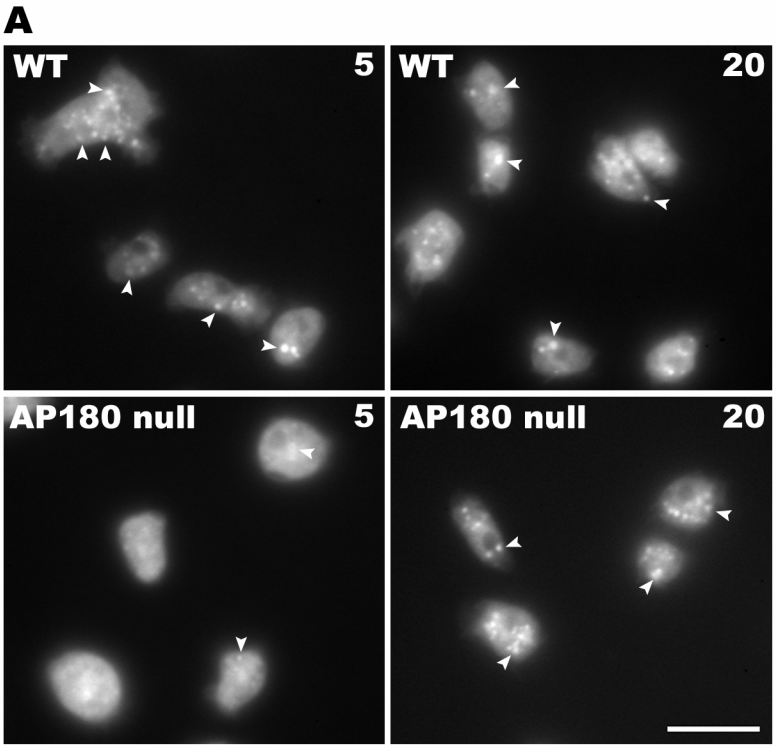


Figure 2.6: AP180 null cells display normal endocytosis and development**.** Fluid phase endocytosis is normal in AP180 null cells. Cells were incubated with FITC-Dextran (2mg/ml). At the indicated times, samples of cells were lysed and quantified for the amount of internalized FITC-Dextran using a fluorometer. AP180 null cells (closed circles) internalized FITC-Dextran at the same rate as wild-type cells (open circles). B. Development of wild-type cells (Ax2) and AP180 null cells. Cells were plated on a lawn of *E. coli*. Following depletion of bacteria, wild-type (Ax2) and AP180 null cells differentiated into fruiting bodies consisting of a robust stalk topped by a round sorus. Scale bar, 0.5 mm C. Spore morphology in wild-type and AP180 null cells. Spores harvested from the sorus of wild-type and AP180 null cells' fruiting bodies displayed the same oblong shape. Scale bar 10 μm.

2.2.8 AP180 null cells display normal lysosomal and post-lysosomal compartments and actin organization.

Dictyostelium is an excellent system to study the endocytic pathway because macropinocytosis and phagocytosis are mechanistically similar to more complex eukaryotic cells. The pathway of endocytosis to exocytosis in *Dictyostelium* occurs rapidly (within an hour) and involves a non-linear progression of fission and fusion events throughout the maturation of the pinosome/endosome [reviewed in (Maniak, 2003)]. During macropinocytosis, the newly formed pinosome is initially neutral in pH, but becomes acidic as it matures and acquires proton pumps. After the pinosome acquires hydrolases and transitions into a lysosome, its acidic pH facilitates the processing of its contents (Souza *et al.*, 1997). As the lysosome matures and acquires different proteins, such as vacuolin, it becomes a post-lysosomal compartment with a neutral pH (Jenne *et al.*, 1998). Clathrin is believed to be involved in recycling and sorting events from the endosomes to the plasma membrane (Neuhaus and Soldati, 1999). Therefore, to examine the role of AP180 in the endocytic pathway we examined two stages; the formation of lysosomes and post-lysosomes. The acidic pH of lysosomes allows for the use of dyes like LysoSensor Green which are detectable by release of fluorescence following their protonation in an acidic environment (Cardelli *et al.*, 1986). Living wild-type and AP180 null cells were incubated in LysoSensor Green and imaged for 20 minutes (Figure 2.7A). After 5 minutes of incubation with the LysoSensor, wild-type cells formed multiple lysosomes. AP180 null cells had fewer visible lysosomes at 5 minutes but at 8 minutes most cells had formed lysosomal compartments (data not shown). The significance of the slight delay in the formation of lysosomes in AP180 null cells still needs to be determined. After 20 minutes of incubation with LysoSensor Green, both wild-type and AP180 null cells formed multiple lysosomal compartments. This result suggests that the

absence of AP180 does not significantly affect the formation of lysosomal compartments in *Dictyostelium*. To examine the formation of post-lysosomal compartments, wild-type, AP180 null cells, wild-type cells expressing GFP-AP180 and AP180 null cells expressing GFP-AP180 were stained with a monoclonal antibody against vacuolin (Figure 2.7 B,C). The formation of post-lysosomal compartments was evident in all cell lines tested (Figure 2.7B). However, cells expressing GFP-AP180 showed an increase in the number of post-lysosomes compared to wild-type and AP180 null cells (Figure 2.7C). In addition, imaging cells stained with Texas-Red labeled phalloidin showed that wild-type and AP180 null mutants shared a similar organization of the actin cytoskeleton (Figure 2.8).



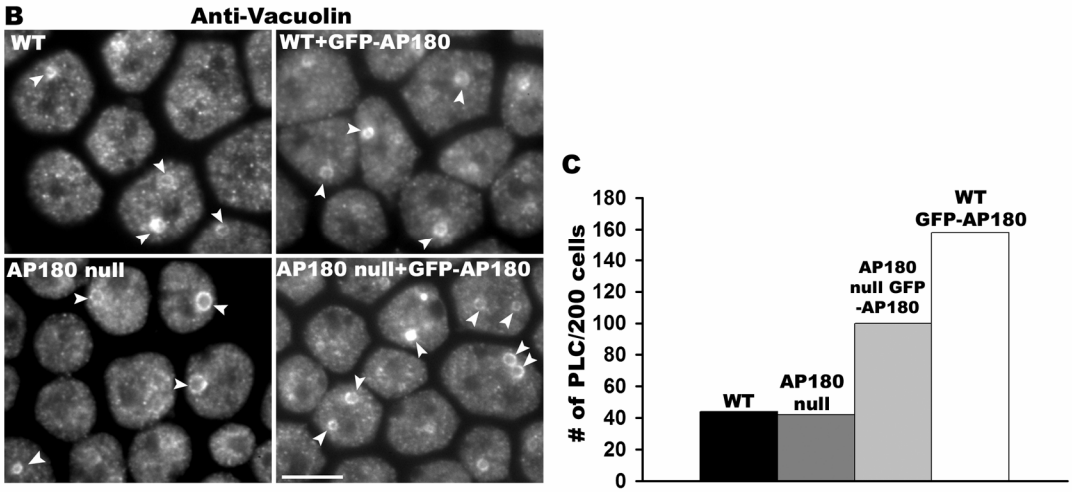


Figure 2.7: Late endocytic pathway in wild-type and AP180 null cells. **(A)** Wild-type (Ax2) and AP180 null cells were incubated in Lysosensor Green and imaged at different time points. In wild-type cells, lysosomal compartments (arrows) became evident after five minutes of incubation with the LysoSensor (top left panel). By twenty minutes both wild-type and AP180 null cells contained multiple lysosomes (number denotes time in minutes). (B,C) Overexpression but not the absence of AP180 causes an increase in post lysosomal compartments (PLC). Cells were gently flattened and stained with a monoclonal antibody against Vacuolin B. Wild-type and AP180 null cells showed about 20 PLC/100 cells whereas wild-type and AP180 null cells expressing GFP-AP180 showed a 4-fold and a 1.5 fold increase respectively. Scale bars 10μm.

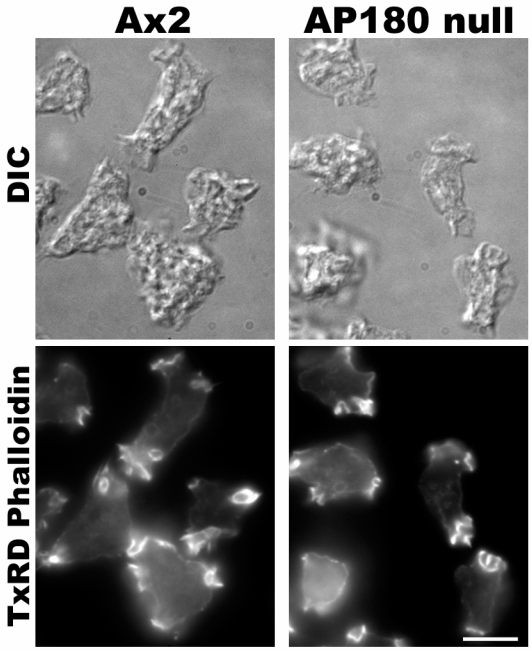


Figure 2.8: Actin organization in wild-type and AP180 null cells. Wild-type (Ax2) and AP180 null cells were fixed and stained with Texas Red Phalloidin to visualize actin. Actin localized to the cortex of the cells and membrane protrusions. The actin organization was unperturbed in the absence of AP180. Scale bar 10μm.

2.2.9 AP180 null cells are osmosensitive

To investigate the role of AP180 in osmoregulation, we examined the behavior of AP180 null cells in a hypo-osmotic environment. I transferred wild-type and AP180 mutant cells from their culture media to water and imaged them using DIC microscopy (Figure 2.9). Wild-type cells adapted quickly to the hypo-osmotic environment by increasing the number and activity of their contractile vacuoles. In contrast, AP180 null cells were osmosensitive. When placed in a hypo-osmotic environment, AP180 null cells initially formed blebs on their membrane, and then formed large contractile vacuoles

(Figure 2.9). Although the enlarged contractile vacuoles persisted after hours in water, the effect was not completely deleterious. Even after several hours in water, AP180 null cells remained viable when they were returned to nutrient growth. The contractile vacuole defect was specific for the absence of AP180 as expressing GFP-AP180 in the null cells rescued this defect (data not shown). The osmosensitive phenotype was reminiscent of a similar defect exhibited by clathrin light chain null cells (Wang *et al.*, 2003). Similar to the response of AP180 null cells, clathrin light chain mutants also formed enlarged contractile vacuoles when shifted to a hypo-osmotic environment. Like those of AP180 null cells, the contractile vacuoles in clathrin light chain mutants were able to fill and discharge, but they grew to a much larger size than the contractile vacuoles of wild-type cells.

We compared the maximum size attained by contractile vacuoles in wild-type and AP180 null cells using time-lapse DIC images of cells in water. In nutrient medium (HL-5) the average maximum diameter of wild-type contractile vacuoles was 2.8 microns (± 0.49, n=27) (Figure 2.10A). When placed in water, the contractile vacuoles of wild-type cells grew larger (3.2 microns, ± 0.43, n=79) and then folded into the plasma membrane as they discharged their contents to the extracellular environment (Figure 2.10B). By comparison, AP180 null cells grown in nutrient medium had contractile vacuoles that were slightly larger than those in wild-type cells (3.4 microns ± 0.52, n=27) (Figure 2.10A). When AP180 null cells were exposed to water, their contractile vacuoles grew substantially larger (Figure 2.10B). Their size reached 4.9 microns (± 0.79, n=79) and a significant number of contractile vacuoles (30%) reached a maximum diameter range of 5.1-5.5 microns.

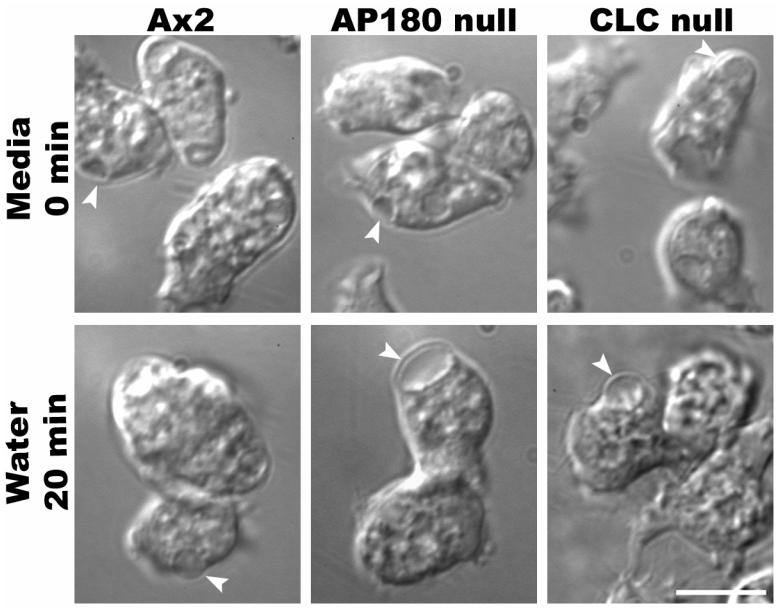


Figure 2.9: AP180 null cells develop large contractile vacuoles in water. Wild-type cells (Ax2), clathrin light null cells (CLC null) and AP180 null cells are shown in HL-5 media (Media, top row) and 20 minutes after shifting cells to water (bottom row). Contractile vacuoles are indicated with arrows. Scale bar, 10 microns.

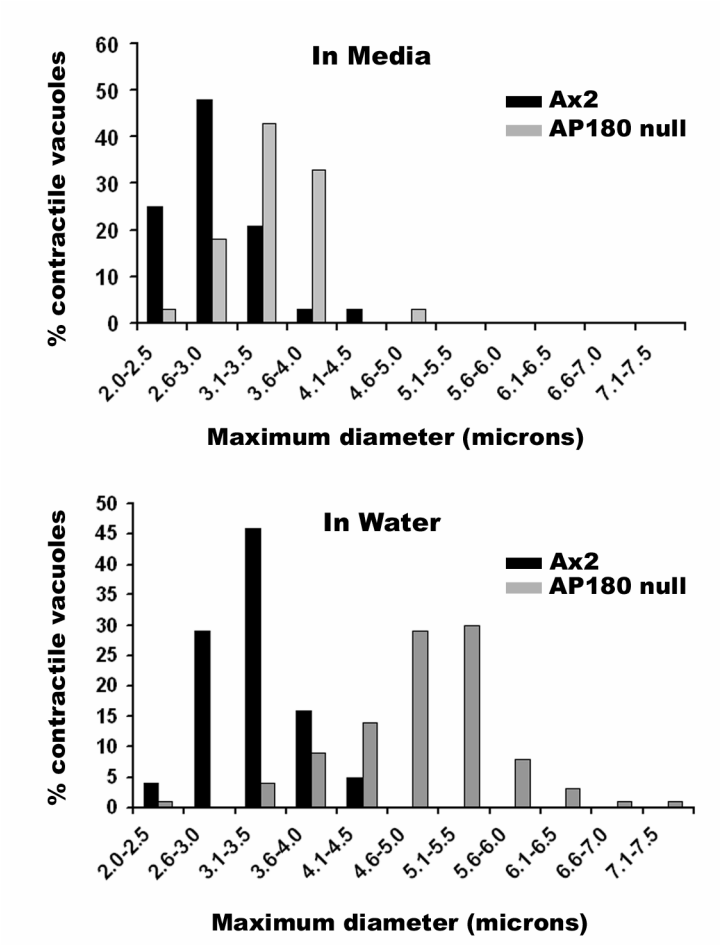


Figure 2.10: Contractile vacuole size in wild-type and AP180 null cells. Living wild-type (Ax2) and AP180 null cells were imaged under DIC optics. The expansion and discharge cycle of contractile vacuoles in media (n=27) and in water (n=79) for each cell line was monitored and the maximum diameter of the vacuole was recorded. In media (top graph), the contractile vacuoles of AP180 null cells were slightly larger than those of wild-type cells. In water (bottom graph) the contractile vacuoles of AP180 null cells were much larger than those of wild-type cells.

2.2.10 The contractile vacuole cycle is prolonged in AP180 mutant cells

To monitor the dynamics of the contractile vacuole in living cells, we transfected wild-type and AP180 null cells with an expression plasmid for Dajumin-GFP, a marker for the contractile vacuole network in *Dictyostelium* (Gabriel *et al.*, 1999). This marker allowed us to visualize the dynamics of the contractile vacuole in real time in a hypo-osmotic environment. We shifted wild-type cells and AP180 null cells expressing Dajumin-GFP from culture media to water, and imaged active contractile vacuoles as they filled and discharged their contents. The contractile vacuole of wild-type cells filled quickly, reached its maximum size and then discharged to the extracellular environment. Examination of contractile vacuole activity in wild-type cells showed an average cycle time of 44 seconds (± 6.9, n=16) (Figure 2.11). In contrast, the activity of the contractile vacuoles of AP180 null cells persisted. After expanding to their maximum diameter, the enlarged contractile vacuoles in AP180 null cells lingered near the plasma membrane, stretched the membrane and then fused and discharged their contents. Examination of contractile vacuole activity in AP180 mutants showed that these contractile vacuoles filled with kinetics similar to wild-type cells. However, since these contractile vacuoles became much larger and were delayed in fusion, the total cycle from filling to expulsion was 90 seconds (± 15.6, n=12), twice as long as the cycle displayed by wild-type cells (Figure 2.11).

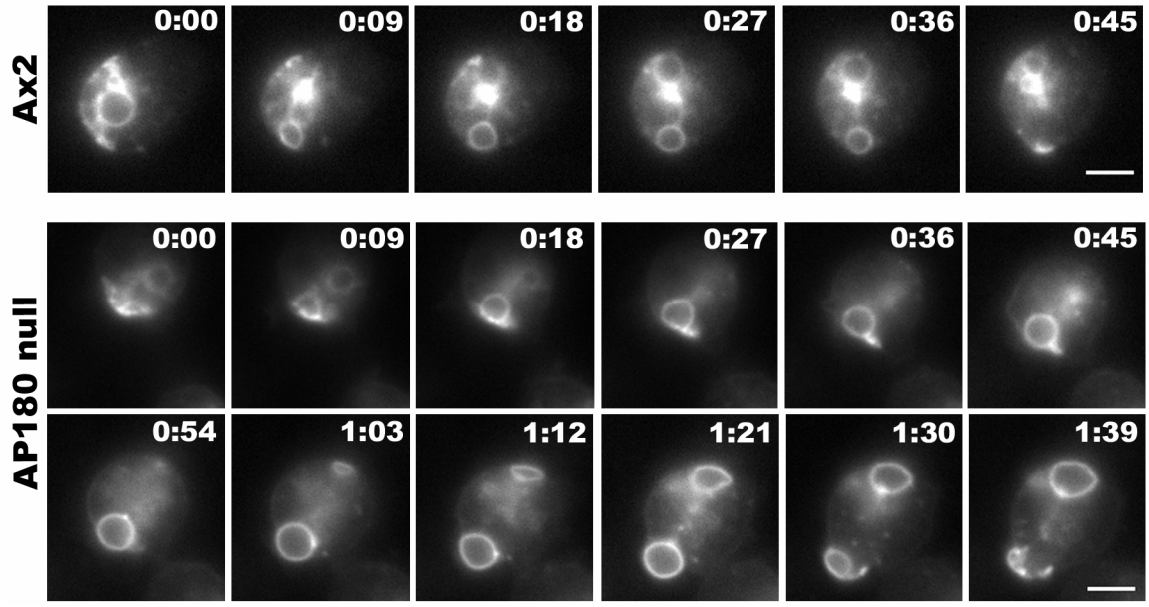


Figure 2.11: Contractile vacuole activity is prolonged in AP180 null cells. Time lapse images of a wild-type cell (Ax2) and an AP180 null cell expressing a contractile vacuole marker, Dajumin-GFP, are shown after incubation in water and imaging with fluorescence optics. In the wild-type cell (Ax2), a contractile vacuole at the lower edge of the cell expands and discharges within 45 seconds. In contrast, the contractile vacuole at the lower edge of the AP180 null cell expands and discharges within 1 minute and 39 seconds. Scale bar, 5 microns.

2.2.11 Drainin localization to the contractile vacuole is not affected in the absence of AP180.

Drainin is a protein involved in osmoregulation in *Dictyostelium* (Becker *et al.*, 1999). GFP-drainin localizes to the contractile vacuole and is thought to be involved in the fusion of the contractile vacuole with the plasma membrane as the contractile vacuole discharges. Evidence for this comes from drainin null cells that show an extremely large

contractile vacuole that fails to discharge (or drain hence the name drainin) (Becker *et al.*, 1999). Therefore it is believed that drainin acts as a volume sensor that controls the size of the contractile vacuole bladder. Because AP180 null cells display enlarged contractile vacuoles, we wanted to examine whether drainin was mislocalized in AP180 null cells (Figure 2.12). Therefore I made use of GFP-tagged drainin (a generous gift from Dr. Gunther Gerisch)(Becker *et al.*, 1999) and transformed it in wild-type Ax2 and AP180 null cells. When wild-type cells were transferred from culture media to water, the contractile vacuoles became active and drainin clearly localized to the contractile vacuole (asterisk). Labeling of the contractile vacuole by drainin was most evident as the contractile vacuole expanded to its maximum size and remained associated until the point of expulsion. Similarly, in AP180 null cells, drainin also localized to the contractile vacuole throughout the cycle of expansion and contraction.

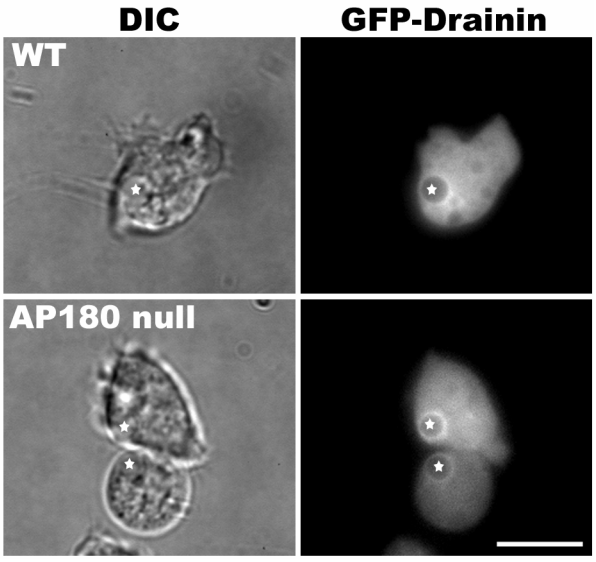


Figure 2.12: GFP-Drainin localizes to the contractile vacuole. Living wild-type and AP180 null cells expressing GFP-Drainin in water. GFP-Drainin labeled the contractile vacuole (asterisk) in both wild-type and AP180 null cells. Scale bar 10μm.

2.2.12 The contractile vacuoles in AP180 null cells contain proton pumps

The multi-subunit proton pump V-H^+ATPase is found predominantly on the contractile vacuole where it is thought to mediate the transport of protons into the lumen of the contractile vacuole (Heuser *et al.*, 1993). The result of this transport is the import of water from the cytoplasm into the lumen of the contractile vacuole and, subsequently, the discharge of excess water in the extracellular environment. To test whether the contractile vacuoles in AP180 null cells contain the vacuolar proton pump as in wild-type cells, I transformed a GFP-tagged subunit of V-H^+ATPase, VatM-GFP (a generous gift from Dr. Margaret Clarke)(Clarke *et al.*, 2002) in wild-type Ax2 cells and AP180 null cells (Figure 2.13). The transformed cells were fixed and visualized using fluorescence microscopy. As was previously reported, in wild-type cells, VatM-GFP localized to vesicular compartments including the contractile vacuole and reticular network (Clarke *et al.*, 2002). The localization of VatM-GFP was not dependent on the presence of AP180 as AP180 null cells displayed the same localization pattern of VatM-GFP as seen in wild-type cells.

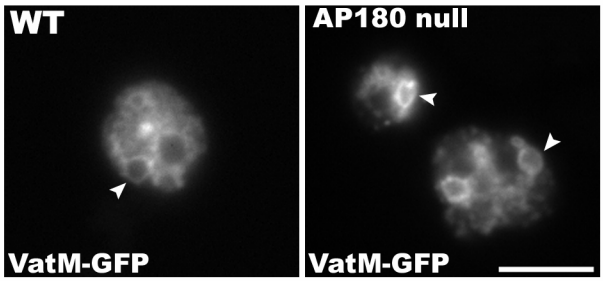


Figure 2.13: VatM-GFP localizes to the contractile vacuole in the absence of AP180. In wild-type and AP180 null cells expressing a subunit of the proton pump V-H^+ATPase, VatM-GFP, the proton pump localized to the contractile vacuole network (arrows). Scale bar 10μm.

2.2.13 AP-1 is required for the normal localization of AP180

The Golgi clathrin adaptor protein, AP-1, is another clathrin related protein that functions in osmoregulation. In mammalian cells, AP-1 bound to AP180 in vitro indicating that AP180 could also function at the Golgi alongside clathrin and AP-1 (Meyerholz *et al.*, 2005). To examine the effect of the absence of AP-1 on the localization of AP180 we fixed wild-type and AP-1μ null cells (Lefkir *et al.*, 2003) expressing GFP-AP180, and visualized them using fluorescence microscopy (Figure 2.14). In wild-type cells GFP-AP180 localized as punctae on the plasma membrane and within the cytoplasm. However, in the absence of the μ-subunit of AP-1, the cytoplasmic GFP-AP180 punctae were significantly reduced and the punctae on the plasma membrane became more prominent. This suggests that AP180 requires the presence of functional AP-1 to localize properly.

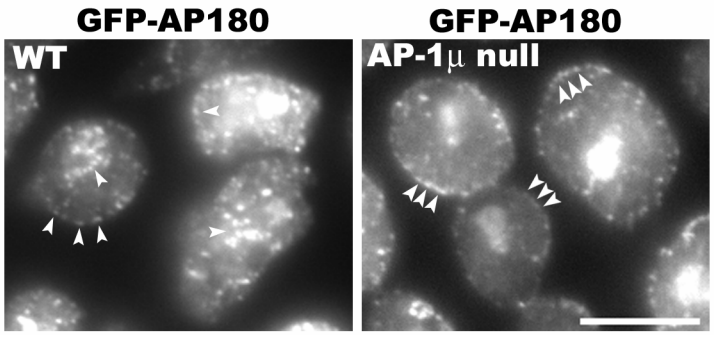


Figure 2.14: GFP-AP180 is mislocalized in the absence of the AP-1μ subunit. Wild-type and AP-1μ null cells expressing GFP-AP180 were fixed and visualized using fluorescence microscopy. In wild-type cells, GFP-AP180 localized to punctae within the cytoplasm and on the plasma membrane (arrows). However, in the AP-1μ null cells the cytoplasmic punctae are significantly reduced and the punctae on the plasma membrane are more prominent. The bright spots represent the nucleus. Scale bar 10μm.

2.3 DISCUSSION

2.3.1 Identification of the *Dictyostelium* AP180 ortholog reveals a new function for an adaptor protein.

In this work, I identified the AP180 ortholog in *Dictyostelium* and examined the contribution of AP180 to clathrin-mediated functions. GFP-AP180 localized as punctae at the plasma membrane, cytoplasm, and the contractile vacuole, and shared extensive colocalization with clathrin at these sites. In clathrin heavy chain null cells, GFP-AP180 continued to localize in punctae on the plasma membrane, but not in cytoplasmic punctae. I attempted to raise an antibody against AP180 using GST-tagged AP180 however the antibody produced was unsuitable for immunofluorescent experiments. In

AP180 null cells, the distribution of clathrin punctae was similar to wild-type cells on the plasma membrane and within the cytoplasm, but was increased on the contractile vacuole. AP180 cells null showed a profound increase in maximum contractile vacuole size. Like clathrin light chain null cells, AP180 null cells exhibited enlarged contractile vacuoles with protracted expansion. These results suggest that AP180 is a specific adaptor for clathrin at the contractile vacuole, and functions with clathrin in regulation of contractile vacuole size.

2.3.2 Relationship between clathrin and AP180 on the plasma membrane

The assembly of clathrin triskelions into ordered lattices on membranes is thought to be promoted by assembly proteins that bind to the plasma membrane through their interactions with phosphoinositides and specific cargo. *In vitro* assembly studies clearly show that AP180 binds PIP2 and that AP180 can efficiently assemble clathrin triskelia into lattices (Lindner and Ungewickell, 1992; Morris *et al.*, 1993; Ye *et al.*, 1995; Ye and Lafer, 1995b; Ford *et al.*, 2001). In *Dictyostelium* clathrin heavy chain null cells, AP180 was clustered into punctae on the plasma membrane. This association of AP180 with the plasma membrane in the absence of clathrin heavy chain is likely driven by the interaction between the ANTH domain of AP180 and the phosphoinositides at the plasma membrane or through interactions between binding motifs within AP180 for other adaptor proteins that reside at the plasma membrane such as AP-2 or EH-domain-containing proteins. The punctae for AP180 suggests that these other proteins might crosslink AP180 even in the absence of clathrin.

Reconstitution of clathrin budding reactions with lipids and purified AP180, epsin, and clathrin show that AP180 is essential for binding clathrin to liposomes while epsin drives curvature of the lattice (Ford *et al.*, 2001; Ford *et al.*, 2002). In contrast, our study of living *Dictyostelium* AP180 null cells showed that clathrin could assemble into

punctae on the plasma membrane even in the absence of AP180. Since the *Dictyostelium* genome contains only a single gene for AP180, other clathrin assembly proteins must drive clathrin assembly on the plasma membrane of AP180 null cells.

2.3.3 AP180 null cells are osmosensitive

The contractile vacuole system functions in osmoregulation, a particularly important adaptation for protists exposed to continuous osmotic changes in their environment. This organelle consists of an interconnected meshwork of tubules and bladders that fill with water, fuse with the plasma membrane, and then contract to discharge water to the extracellular milieu. Normally, wild-type cells growing in culture media contain a few moderately active contractile vacuoles that maintain the osmotic balance of the cell. When the extracellular environment changes from iso-osmotic to hypo-osmotic, the number of contractile vacuoles and their activity increase to cope with increased osmotic pressure and thus prevent the cell from swelling and bursting. Among the fascinating features of the contractile vacuole is its ability to fill the bladder to a discrete size every time it goes though the cycle of expanding and contracting. The mechanism for the control of size for the contractile vacuole bladder is not known. As the bladder fills with water, the tubules connected to the bladder shorten as they are incorporated into the expanding bladder. Following the emptying of the bladder, the tubules elongate to regenerate the contractile vacuole system and repeat the cycle (Gerisch *et al.*, 2002; Heuser, 2006).

Clathrin is required for a functional contractile vacuole because clathrin heavy chain mutant cells contain a dispersed contractile vacuole system without tubules and round up and burst in the presence of water (O'Halloran and Anderson, 1992b). Similarly, clathrin light chain null cells are also osmosensitive and display enlarged contractile vacuoles (Wang *et al.*, 2003). Our results suggest that AP180 and clathrin cooperate in

the cycle of contractile vacuole activity because of two observations: (i) the contractile vacuole phenotype shared between AP180 null cells and clathrin light chain null cells; and (ii) AP180 and clathrin are each found at the contractile vacuole. Both clathrin light chain and AP180 null cells show enlarged contractile vacuole bladders and prolonged contractile vacuole cycles. These deficiencies could be caused by defective clathrin lattices assembled without AP180 on the contractile vacuole. AP180 null cells continued to target clathrin on the contractile vacuole. Indeed, clathrin localized even more prominently on the contractile vacuoles of AP180 null cells. This increase in clathrin could reflect an increased time for clathrin assembly without AP180, in view of in vitro studies that AP180 increases the efficiency of clathrin assembly into lattices (Hao *et al.*, 1999). It is also possible that clathrin forms imperfect lattices on the contractile vacuoles of AP180 null cells, as shown recently for mammalian cells (Meyerholz *et al.*, 2005). If clathrin vesicles must form efficiently into structured lattices on the contractile vacuole for full function, then inefficient clathrin assembly could account for the delayed cycle of the contractile vacuole seen in AP180 null cells.

How do clathrin and AP180 contribute to contractile vacuole function? One possibility is that AP180 null cells fail to sort proteins that are important for fusion of the contractile vacuole. The mechanism for the fusion of the *Dictyostelium* contractile vacuole with the plasma membrane is not known, but conceivably the fusion of this organelle could be regulated by SNARE proteins. In synapses, AP180 is thought to selectively retrieve a synaptic vesicle v-SNARE, synaptobrevin, into clathrin coated vesicles, and AP180 mutants do not localize this v-SNARE properly (Nonet *et al.*, 1999; Bao *et al.*, 2005). By analogy, AP180 could function in *Dictyostelium* by retrieving a v-SNARE important for contractile vacuole fusion. In the absence of AP180, the regenerating contractile vacuole might lack sufficient v-SNARES for efficient fusion and

consequently would expand to an abnormally large size. Alternatively, the presence of clathrin and AP180 assembled into punctae on the contractile vacuole suggests that AP180-associated coated vesicles function on the contractile vacuole membrane itself, perhaps by remodeling and preparing the contractile vacuole membrane so that it can fuse with the plasma membrane and efficiently discharge its contents.

The finding that clathrin light chain null cells showed a diminished association of AP180 with the contractile vacuole may seem paradoxical. The standard view is that assembly proteins bind to the plasma membrane first and then recruit clathrin. However the decrease in AP180 localization on the contractile vacuole of clathrin light chain cells suggests that clathrin could also influence AP180 distribution on membranes. It is possible that coated pits are built by the dynamic interaction of clathrin triskelions and AP180 proteins, as each recruits the other to stabilize the growing clathrin lattice. Without light chains, clathrin triskelia are crippled in function (Wang *et al.*, 2003), and perhaps these compromised triskelia are unable to stabilize AP180 on the contractile vacuole. Thus the interplay between AP180 and clathrin to build a stable and regular lattice on this membrane could be impaired in clathrin light chain null cells. Clearly, my results support current views that different clathrin-mediated trafficking pathways require different adaptor proteins.

Chapter 3: Collaboration between AP-2 and AP180 in clathrin assembly and contractile vacuole function in *Dictyostelium*

3.1 INTRODUCTION

Clathrin mediated endocytosis requires an array of endocytic proteins that function in concert to ensure the proper internalization and sorting of receptors from the plasma membrane (Kirchhausen, 1999; Brodsky *et al.*, 2001). The assembly of clathrin triskelions into lattices on the plasma membrane is promoted by adaptor proteins. Adaptor proteins and other endocytic accessory proteins form a complex scaffold that connects clathrin lattices with specific cargo on the plasma membrane (Boehm and Bonifacino, 2001). The most abundant adaptor protein in clathrin coated vesicles on the plasma membrane is the heterotetrameric protein AP-2, which functions both to assemble clathrin and to select cargo (Owen *et al.*, 2004). AP-2 is composed of 4 different subunits, , 2, μ2 and 2 (Kirchhausen, 1999). The and 2 subunits, or large subunits, confer binding to other adaptor and accessory proteins of the endocytic pathway while 2 also binds and assembles clathrin (Collins *et al.*, 2002; Kirchhausen, 2002; Mousavi *et al.*, 2004). The medium subunit, μ2 is involved in cargo recognition through a tyrosine-type sorting signal found in the cargo (Owen and Evans, 1998; Bonifacino and Traub, 2003). AP-2 also binds to phosphotidylinositol 4, 5-bisphosphate (PtdIns[4,5]P_2) on the plasma membrane through the and μ2 subunits (Beck and Keen, 1991; Voglmaier *et al.*, 1992; Gaidarov *et al.*, 1996; Rohde *et al.*, 2002). The function of the 2 subunit remains largely unknown although it is thought to link the other subunits together. (Collins *et al.*, 2002). Therefore AP-2 serves not only to link the membrane, the cargo to be internalized

and clathrin, but also as an anchor for other endocytic proteins that function during endocytosis.

The importance of AP-2 for endocytosis has been highlighted by different experimental approaches. An RNAi knockdown of various subunits of AP-2 in HeLa cells decreases the association of clathrin with the plasma membrane and inhibits the endocytosis of the transferrin receptor and Epidermal Growth Factor (EGF) receptor but not the Low Density Lipoprotein (LDL) receptor or the EGF receptor if cells are incubated in EGF at 4°C prior to measuring internalization rates (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003); (Huang *et al.*, 2004). The discrepancies between the two earlier studies and the one completed by Huang et al were due to the method used to measure internalization rates of EGFR and the high concentration of EGFR used in the study done by Hinrichen et al that favors clathrin-independent uptake. Furthermore, mutation in the 2 subunit of the *Drosophila melanogaster* AP-2 and disruption of the μ2 subunit in mice causes lethality (Gonzalez-Gaitan and Jackle, 1997; Mitsunari *et al.*, 2005).

AP-2 has been shown to assemble clathrin triskelia into clathrin cages (Keen, 1987; Keen *et al.*, 1987; Lindner and Ungewickell, 1992; Hao *et al.*, 1999) and to bind other important assembly proteins such as AP180 (Hao *et al.*, 1999; Owen *et al.*, 2000).

AP180 is another significant player implicated in the efficient assembly of uniform sized clathrin cages (Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988; Ye and Lafer, 1995a). A conserved N-terminal ANTH domain confers binding of AP180 to PtdIns(4,5)P_2 and recruitment to the plasma membrane (Ford *et al.*, 2001). AP180 binds directly to clathrin through conserved motifs, DLL and/or L(L,I)(D,E,N)(L,F)(D,E) (Dell'Angelica *et al.*, 1998; Morgan *et al.*, 2000; ter Haar *et al.*, 2000) and to the 2 appendage domain of AP-2 though a DPF/W motif (Owen *et al.*, 1999; Owen *et al.*, 2000). AP180 assembles clathrin monomers into lattices about four times better than AP-

2 alone (Lindner and Ungewickell, 1992) but combined with AP-2 their assembly activity is greater that either protein alone (Hao *et al.*, 1999). RNAi studies suggest that AP180 is involved in the uptake of EGFR but not of the transferrin receptor in HeLa cells (Huang *et al.*, 2004). This points to different uses of adaptor proteins for specific cargos internalized via clathrin coated vesicles.

Currently, AP-2 and AP180 are thought to work together in at least some clathrin coated pits to assemble clathrin and to select specific and possibly distinct (for example the transferrin receptor) cargo from the plasma membrane for internalization. In support of this model, in vitro studies show direct binding between AP-2 and AP180, (Hao *et al.*, 1999) and immunofluorescent images show colocalization of the two proteins into punctae (Hinrichsen *et al.*, 2003; Meyerholz *et al.*, 2005). However, a reduction in AP-2 in HeLa cells only moderately reduces the membrane association of AP180/CALM (Hinrichsen *et al.*, 2003). In yeast, deletion of all 13 genes that encode for the adaptor proteins as well as the two genes encoding AP180 did not affect clathrin function (Huang *et al.*, 1999). To date, the relationship between these two adaptors and clathrin remains unresolved.

I have previously shown that *Dictyostelium* AP180 interacts with clathrin and is involved in the regulation of contractile vacuole size (Stavrou and O'Halloran T, 2006). Our lab has shown that AP-2 is a unique clathrin adaptor in *Dictyostelium* in that it affects the recruitment of clathrin to the plasma membrane (Wen and O'Halloran, personal communication). *Dictyostelium* AP-2 is also important in cytokinesis and chemotaxis (Wen and O'Halloran, personal communication). The absence of AP-2 does not affect the co-localization of AP180 with clathrin at the plasma membrane (Wen and O'Halloran, personal communication) indicating that these two adaptors might associate with clathrin independently of each other. Furthermore, in clathrin heavy chain null cells,

cytoplasmic punctae are lost, but AP-2 and AP180 remain associated in punctae on the plasma membrane. This suggests that AP-2 and AP180 do not require clathrin to associate at the plasma membrane but they require clathrin in order to be internalized into coated vesicles in the cytoplasm.

In the present study, we examined how AP-2 and AP180 function together to recruit clathrin at the membrane and the contractile vacuole. We deleted the genes for both AP-2 and AP180 to develop a double null *Dictyostelium discoideum* cell strain. We found that in the absence of AP-2 and AP180, the association of clathrin with the plasma membrane and the contractile vacuole was significantly reduced. The contractile vacuole in these null mutants was enlarged and a contractile vacuole marker was mislocalized to the plasma membrane. Our results show that AP-2 and AP180 function redundantly to recruit clathrin at the plasma membrane and cooperatively in the regulation of contractile vacuole activity.

3.2 RESULTS

3.2.1 AP-2 and AP180 cluster in the same punctae on the plasma membrane

AP-2 and AP180 are both clathrin assembly proteins that localize to the plasma membrane and both bind and assemble clathrin into lattices. To examine whether these two assembly proteins share a common location on the plasma membrane, we expressed GFP-tagged AP180 in cells and stained these cells with an antibody against the alpha subunit of AP-2 (AP-2) (Figure 3.1). AP180 distributed as punctae on the plasma membrane, within the cytoplasm and in a perinuclear area whereas most AP-2 localized as bright punctae on the plasma membrane with fewer dimmer punctae within the cytoplasm. On the plasma membrane most AP180 clustered in the same punctae as AP-2

suggesting that the two proteins could function together in the same coated pit on the plasma membrane. In addition, many AP-2 punctae on the plasma membrane did not contain AP180. This is in contrast to mammalian cells where there is almost complete colocalization between these two proteins (Meyerholz *et al.*, 2005). While AP180 punctae in the cytoplasm were more abundant than AP-2 punctae, occasionally these punctae also contained AP-2.

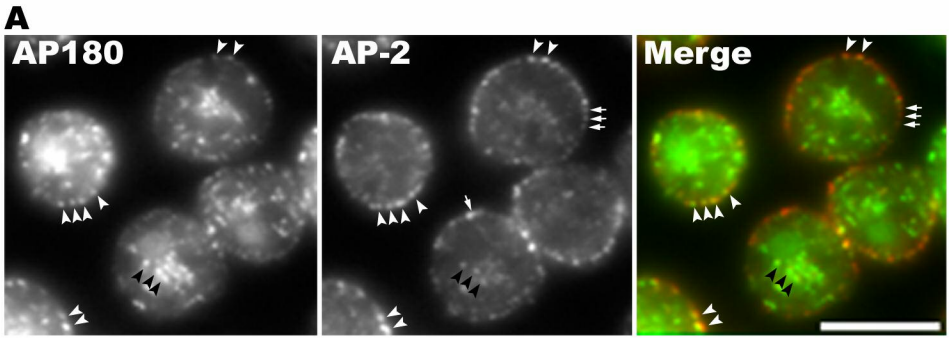


Figure 3.1: AP-2 and AP180 colocalize. Wild-type (DH1) cells expressing GFP-AP180 were stained with an antibody against the -subunit of AP-2 conjugated to TxRD secondary antibody. AP180 clustered on the plasma membrane, the cytoplasm and the perinuclear area (left panel). AP-2 formed punctae predominantly on the plasma membrane and fewer dim punctae in the cytoplasm (middle panel). The merge picture shows that AP-2 (red) and AP180 (green) colocalized on the plasma membrane (arrow heads) and occasionally in the cytoplasm (black arrow heads). AP-2 punctae that did not cluster with AP180 were also found on the plasma membrane (middle and right panels – white arrows) (by Yujia Wen). Scale bar 10μm.

3.2.2 The absence of AP-2 does not affect the localization of AP180 and its association with clathrin

AP180 is a strong clathrin assembly protein and is thought to function with AP-2 to link clathrin to specific sites on the plasma membrane. To examine whether AP180 can associate with the plasma membrane in the absence of AP-2 we examined wild-type and AP-2 null cells expressing GFP-AP180 (Figure 3.2 upper left and bottom left panels). The distribution of AP180 was similar in AP-2 null, and in wild-type cells. In both strains, GFP-AP180 localized as punctae on the plasma membrane, within the cytoplasm and in the perinuclear area. To examine whether AP180 and clathrin continued to cluster within the same punctae, we immunostained wild-type and AP-2 null cells expressing GFP-AP180 with an antibody against clathrin (Figure 3.2). In wild-type and mutant cells, AP180 and clathrin colocalized extensively in the same punctae.

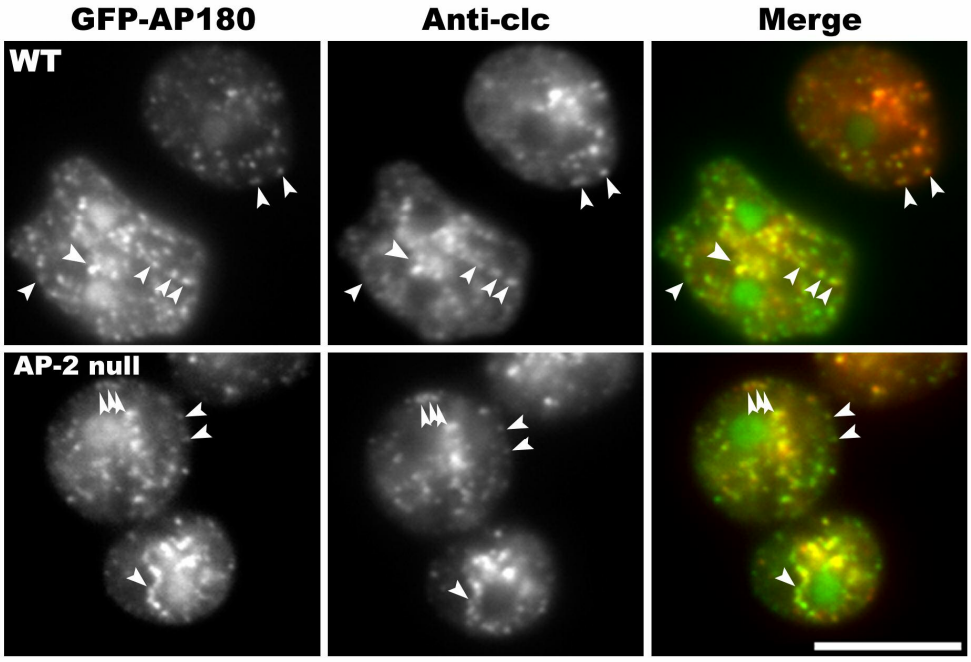


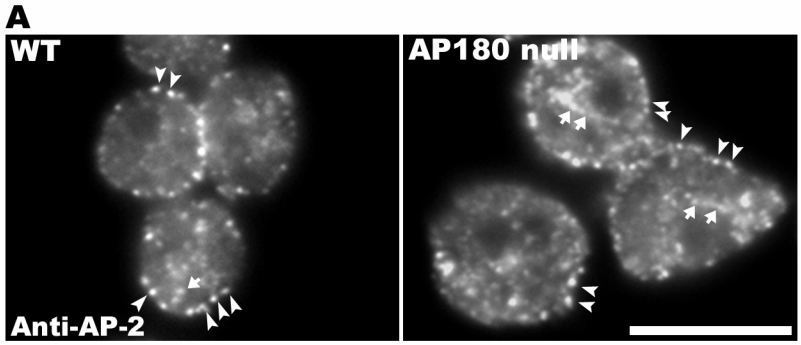
Figure 3.2: The absence of AP-2 does not affect the association of AP180 with clathrin. Wild-type (DH1) and AP-2 null cells expressing GFP-AP180 were fixed and visualized using fluorescence microscopy. AP180 localized as punctae on the plasma membrane, in the cytoplasm and in a perinuclear area (top left panel, arrow heads). This localization was unperturbed in AP-2 null cells. Wild-type and AP-2 null cells expressing GFP-AP180 and stained with an antibody against clathrin light chain conjugated to TxRD revealed that many punctae contained AP180 (green) and clathrin (red) (arrow heads). Scale bar 10μm.

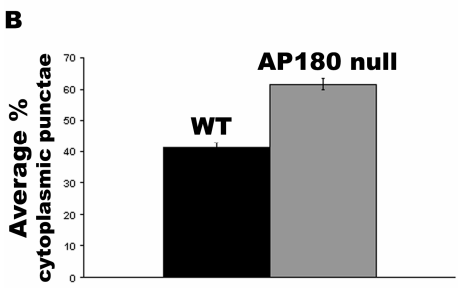
3.2.3 AP-2 remains associated with the plasma membrane and clathrin in AP180 null cells but cytoplasmic punctae increase

To examine the cellular distribution of AP-2 in AP180 null cells we stained wild-type and AP180 null cells with an antibody against AP-2 and examined the cells using immunofluorescence microscopy (Figure 3.3A). While the total number of AP-2 punctae in wild-type and AP180 null cells was similar (wild-type = 28±0.21, n=43; AP180 null cells =33±1.57, n=61 (mean punctae/focal plane ± Standard error [SE]), the distribution of AP-2 punctae was different in the two cell lines. Wild-type cells had ~60% of bright AP-2 punctae on their plasma membrane whereas AP180 null cells had fewer (~40%) punctae on their plasma membrane (Figure 3.3B).

To examine whether clathrin remained associated with AP-2 punctae in AP180 null cells, I stained wild-type and AP180 null cells with a clathrin antibody and imaged the cells with fluorescence microscopy. In both wild-type and AP180 mutants, most of the clathrin punctae seen on the plasma membrane colocalized with AP-2 punctae (Figure 3.3C). In wild-type cells, the cytoplasmic AP-2 punctae occasionally colocalized with

clathrin. However, in AP180 null cells, the increased number of cytoplasmic AP-2 punctae colocalized extensively with clathrin.





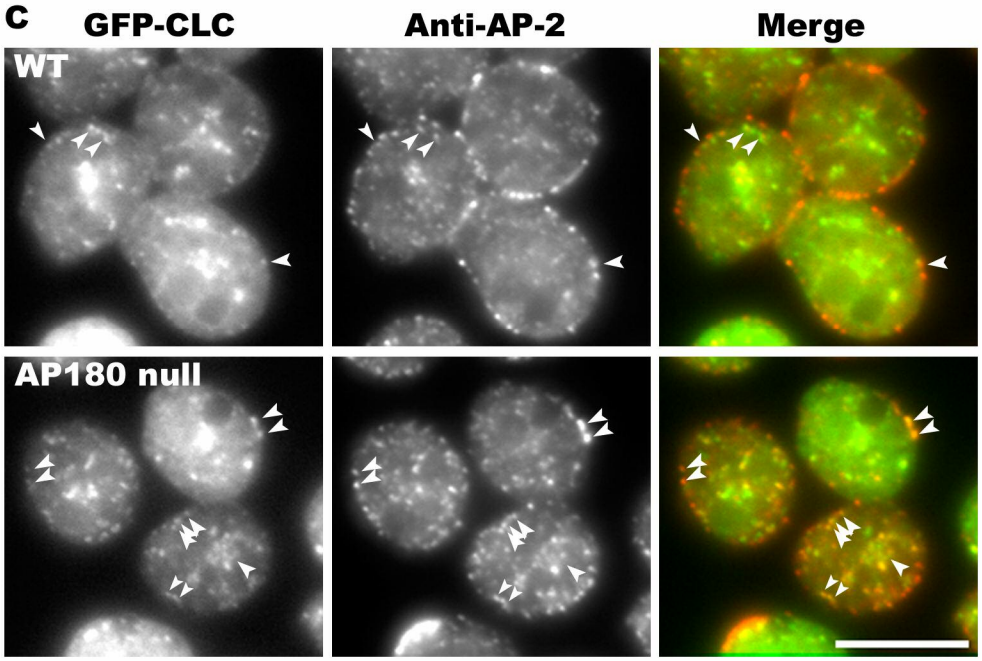


Figure 3.3: Cytoplasmic AP-2 punctae increase in the absence of AP180. (A). Wild-type and AP180 null cells were fixed and stained with an antibody against the -subunit of AP-2. In wild-type cells, AP-2 predominantly localized to the plasma membrane (left panel – arrow heads) with dimmer AP-2 punctae in the cytoplasm (arrows). In the absence of AP180 (right panel) more cytoplasmic AP-2 punctae were observed (arrows). (B). Cytoplasmic and plasma membrane-associated punctae were quantified (WT, n=1214 punctae; AP180 null cells, n=2012 punctae). (C). Cells expressing GFP-clc were stained with an antibody against AP-2. In wild-type cells, clathrin punctae localized to the plasma membrane and cytoplasm (top left panel – arrowheads) whereas AP-2 localized to bright punctae on the plasma membrane and dimmer punctae in the cytoplasm (middle panel – arrowheads). In the absence of AP180, AP-2 and clathrin clustered together both on the plasma membrane and the cytoplasm (by Yujia Wen). Scale bars 10μm.

3.2.4 AP-2 /AP180 double null cell have an enlarged contractile vacuole in media and water

Since both AP180 and AP-2 are clathrin assembly proteins found on the plasma membrane, we examined the possibility that the two proteins share redundant cellular functions. We developed an AP-2 /AP180 double mutant cell line in the *Dictyostelium* DH1 wild-type background by deleting the genes for AP-2 and for AP180. The absence of the products of the AP-2 and AP180 genes was confirmed using western blot analysis (Figure 3.4A).

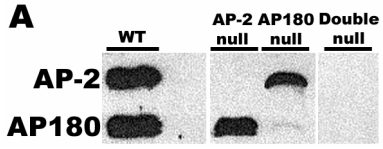
To examine the effect of the AP-2 /AP180 deletion on osmoregulation, we monitored their contractile vacuole activity in different osmotic environments (Figure 3.4B). When wild-type cells in isotonic nutrient media were imaged using Differential Interference Contrast (DIC) microscopy, few contractile vacuoles were seen. These few

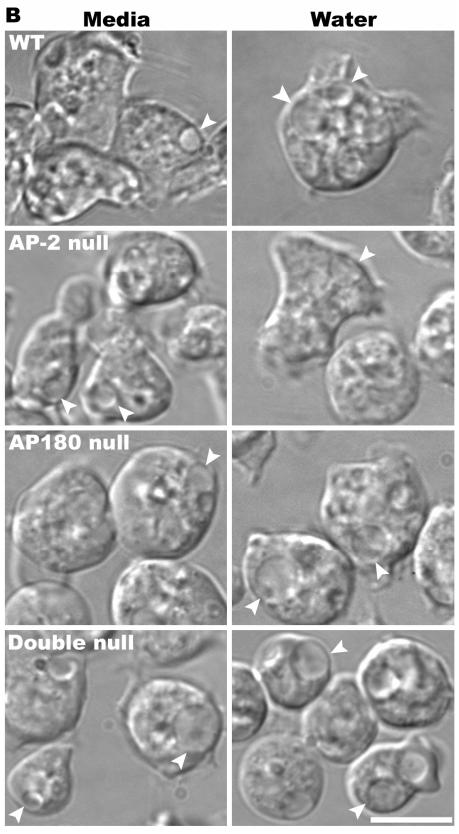
contractile vacuoles became round as they filled, reached a maximum size and moved to the membrane to discharge their contents. In isotonic media, the contractile vacuoles of AP180 null and AP-2 null cells were slightly larger but displayed normal contractile vacuole activity (Figure 3.4B, C). In contrast, even in isotonic media the contractile vacuoles AP-2 /AP180 double null cells were much larger. These contractile vacuoles filled to an abnormally large size and lingered near the plasma membrane before they eventually discharged.

When cells were transferred into water, the contractile vacuoles of wild-type, AP-2 null cells and AP180 null cells became more prominent. While the contractile vacuoles in AP-2 null cells were slightly larger than wild-type, they expanded and contracted similarly to those in wild-type cells without the "lagging" phase seen in the double null mutants. When AP180 null cells were transferred from their media to water, their contractile vacuoles became enlarged as previously reported (Stavrou and O'Halloran T, 2006). When filled, the contractile vacuoles were larger in AP180 null cells than in AP-2 null or wild-type cells. When AP-2 /AP180 double null cells were transferred to water, their contractile vacuoles remained the same large size and continued to linger near the plasma membrane before discharging.

To further explore the defect in contractile vacuole size in these cells we measured the diameter of contractile vacuoles at their maximum using time-lapse DIC images of cells (Figure 3.4C and table 3.1). In nutrient medium, the maximum average diameter of wild-type cells was 3.3±0.58 μm (mean ± SE, n=9), of AP-2 null was 3.4 ±0.61 μm (n=10), of AP180 null cells was 4.0 ±1.08 μm (n=28) and of AP-2 /AP180 double null cells was 4.8±1.08 μm (n=24). In water, the maximum average diameter of contractile vacuoles in wild-type cells remained almost the same (3.4 ±0.65 μm, n=52) whereas those of AP-2 null and AP180 null cells grew larger (4.0 ±0.82 μm, n=40 and

4.2 ±0.73 μm, n=61 respectively). Similar to their size in nutrient media, the contractile vacuoles of AP-2 /AP180 double null cells were abnormally large with an average maximum diameter of 5.2 ±0.81 μm (n=56) and more than half (55%) growing larger than 5.0 μm. Thus AP-2 /AP180 double null cells displayed enlarged contractile vacuoles in both nutrient media and water indicating that the contractile vacuole in AP-2 /AP180 double null cells becomes enlarged independent of the osmotic environment surrounding the cells.





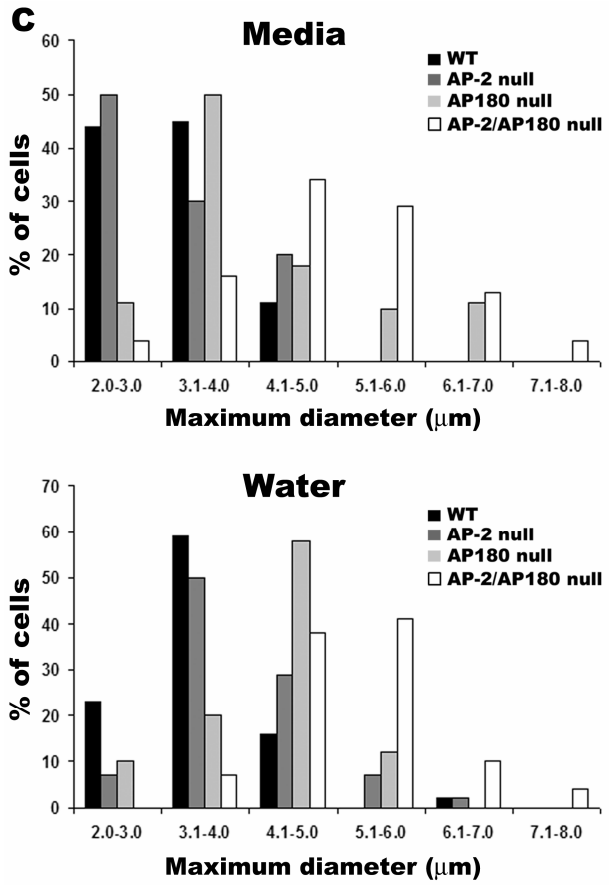


Figure 3.4: AP-2α/AP180 double null cells are osmosensitive. (A). A *Dictyostelium* cell strain carrying deletions in the genes for both AP-2 and AP180 was constructed using homologous recombination. The absence of AP-2 and AP180 protein expression was confirmed by western blot analysis (by Yujia Wen). (B,C). Osmoregulation of wild-type, AP180 null, AP-2 null and AP-2 /AP180 double null cells using DIC microscopy. The cycle of expansion and discharge of contractile vacuoles in media and in water for each cell line was examined and the maximum diameter of the vacuole was scored. In media and water, the contractile vacuoles of AP-2 /AP180 double null cells were much larger than those of wild-type, AP180 null and AP-2 null cells (left panels and graphs). Scale bar 10μm.

Table 3.1: Average maximum contractile vacuole diameter.

	Average maximum diameter (microns)	
Cell line	Media	Water
Wild-type	3.3±0.58	3.4±0.65
AP-2 null	3.4±0.61	4.0±0.82
AP180 null	4.0±1.08	4.2±0.73
AP-2 /AP180 double null	4.8±1.08	5.2±0.81

3.2.5 AP-2 /AP180 double null cells mislocalized a contractile vacuole marker

To image contractile vacuole activity in living cells, we transformed cells with a plasmid expressing Dajumin-GFP which is a contractile vacuole marker (Gabriel *et al.*, 1999). Cells expressing Dajumin-GFP were imaged using fluorescence microscopy (Figure 3.5). In water, where the contractile vacuoles are most active, wild-type cells displayed a dynamic reticular network of tubules and bladders. The tubular network was connected to round bladders and the tubules were incorporated into the bladders as they expanded. AP-2 and AP180 null cells displayed a similar organization of the contractile vacuole network, with bladders and tubules labeled with Dajumin-GFP. In water, the AP-2 /AP180 double null cells showed Dajumin-GFP associated with the contractile vacuole. These cells had only a modest tubular network as if all the tubules had merged into the enlarged contractile vacuole bladder. Moreover, the contractile vacuole marker also uniformly stained the periphery of the cell, a pattern not seen in wild-type cells.

The cycle of expansion and contraction was determined for wild-type, AP-2 , AP180 and AP-2 /AP180 null cells using timelapse images of living cells in water. The average time taken for a complete cycle for wild-type contractile vacuoles was

50±6.4seconds (mean ± SE, n=12), of AP-2 null cells was 79±9.5 seconds (n=11), of AP180 null cells was 84±8.3seconds (n=15) and that of AP-2 /AP180 null cells was 110±8.4seconds (n=14). Therefore, these results indicate that the contractile vacuole in AP-2 /AP180 null cells have a delayed cycle of expansion and contraction because they expand to an abnormally large size.

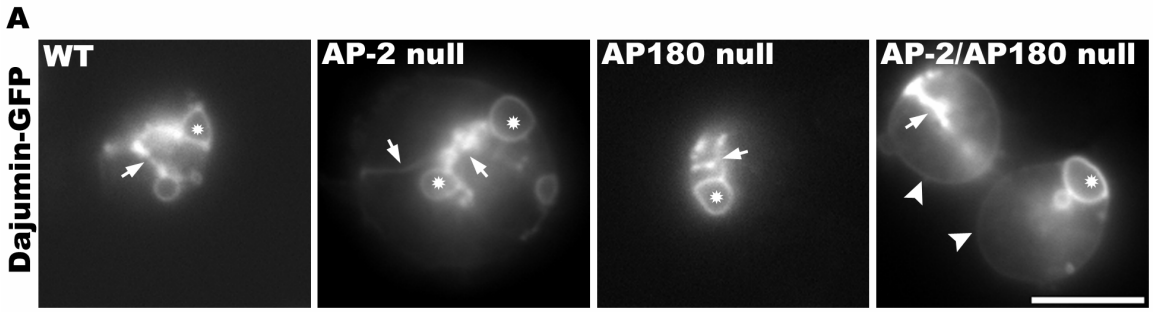
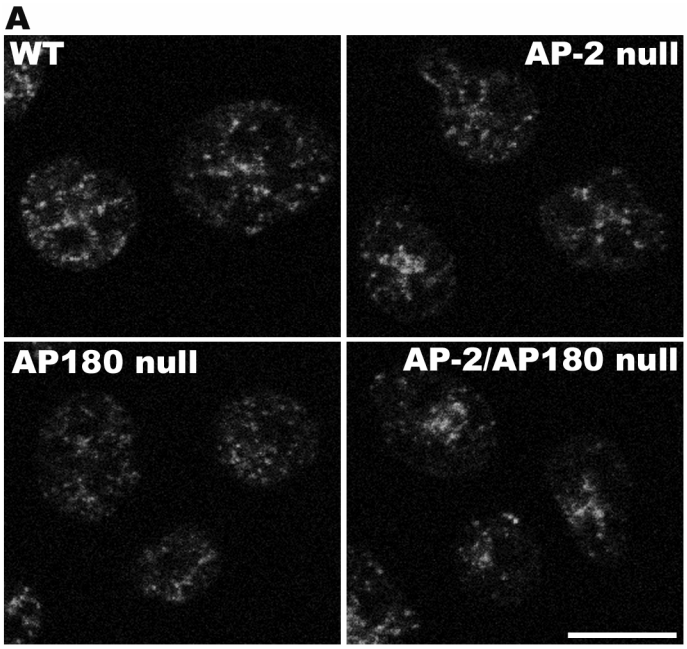


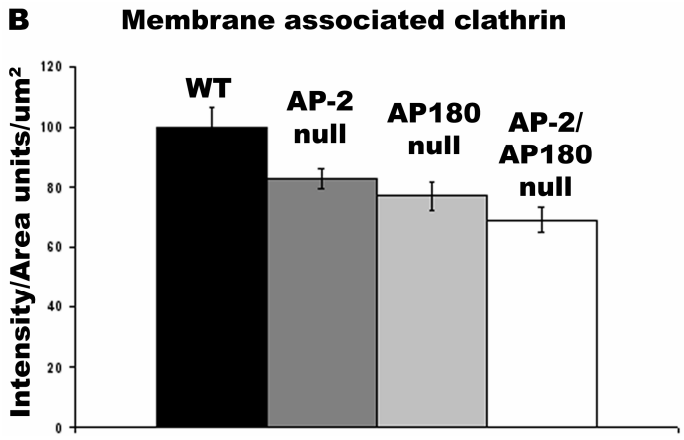
Figure 3.5: A contractile vacuole marker is mislocalized to the cells periphery in the AP-2α/AP180 null cells. The contractile vacuole in wild-type, AP-2 null, AP180 null and AP-2 /AP180 null cells was visualized in living cells expressing Dajumin-GFP, a contractile vacuole marker. In wild-type, AP-2 and AP180 null cells Dajumin-GFP labeled exclusively the contractile vacuole bladders (asterisk) and tubules (arrows). In AP-2 /AP180 double null cells however, this contractile vacuole marker was also redistributed to the plasma membrane (arrowheads-far right panel), a pattern rarely seen in wild-type cells. Scale bar 10μm.

3.2.6 The membrane association of clathrin is reduced in the absence of AP-2 and AP180

AP-2 and AP180 synergistically promote maximum clathrin assembly activity in vitro (Hao *et al.*, 1999).To examine how the absence of two major adaptor proteins would

affect the association of clathrin with the plasma membrane in living cells, I stained wild-type, AP-2 null, AP180 null and AP-2 /AP180 double null cells with an antibody against clathrin, and imaged the cells using confocal microscopy (Figure 3.6A). In wild-type, AP180 null and AP-2 null cells clathrin localized as punctae at the periphery of the cells. However in AP-2 /AP180 double null cells fewer clathrin punctae were seen. To quantify clathrin punctae at the plasma membrane, we quantified the intensity of fluorescence in confocal sections focused on the plasma membrane. We found a 17% and 23% reduction in clathrin association with the plasma membrane in AP-2 and AP180 single knockout cells respectively. In the AP-2 /AP180 double null cells we found 31% decrease in clathrin at the plasma membrane. While the mutants showed diminished clathrin assembled on their plasma membrane, the amount of clathrin expressed in the mutant lines was similar to wild-type cells (Figure 3.6C). These results indicated that clathrin has lost some but not all of its ability to associate with the plasma membrane in the absence of both AP-2 and AP180.


A
WT
AP-2 null
AP180 null
AP-2/AP180 null


B
Membrane associated clathrin
Intensity/Area units/um²
120
100
80
60
40
20
0
WT
AP-2 null
AP180 null
AP-2/ AP180 null

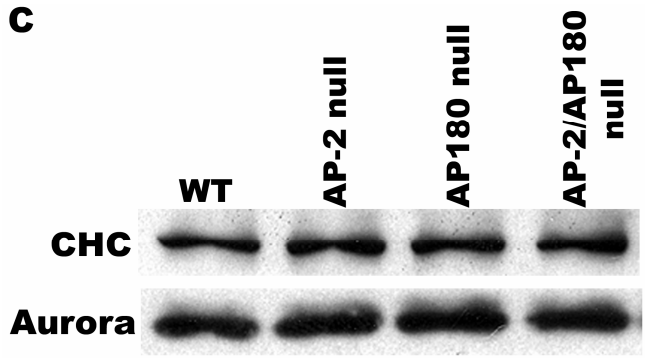

C
WT
AP-2 null
AP180 null
AP-2/AP180 null
CHC
Aurora

Figure 3.6: Reduction of membrane-associated clathrin in AP-2a/AP180 double null mutants**.** (A). Wild-type, AP-2 null, AP180 null and AP-2 /AP180 double null cells were fixed and stained with an antibody against clathrin light chain followed by TxRD conjugated secondary antibody. The images are focused on the top of the cells in order to compare clathrin association at the plasma membrane in the different cells lines. (B). Clathrin punctae were quantified in each cell line as indicated (WT, n=91; AP-2 null, n=100; AP180 null, n=100; n=100) (C). Clathrin protein levels from whole cell lysate from wild-type, AP-2 null, AP180 null and AP-2/AP180 double null was analyzed using western blot analysis. Anti-clc antibody was used to detect the levels of endogenous clathrin and anti-aurora antibody was used as a control (by Yujia Wen). Scale bar 10μm.

3.2.7 The association of clathrin with the contractile vacuole is reduced and altered in the absence of AP-2 and AP180

In *Dictyostelium* cells, clathrin is frequently found on the membranes of contractile vacuoles (Stavrou and O'Halloran T, 2006). To examine the association of clathrin with the contractile vacuole in mutant cells, we imaged living cells expressing GFP-tagged clathrin light chain (clc) in water and followed the association of clathrin with the contractile vacuole (Figure 3.7A). In wild-type, AP-2 and AP180 null cells, punctae of clathrin outlined the circumference of the bladder and frequently remained until the bladder discharged. More than half of the contractile vacuoles in these strains were labeled with clathrin for at least a portion of their cycle (Figure 3.7B). However, in AP-2 /AP180 null cells this pattern was very different. Clathrin was associated with the contractile vacuole bladder in only 24% (n=74) of the double mutants. In the remaining cells (76%), clathrin punctae were clumped to one side of the contractile vacuole bladder.

This dramatic reduction indicates that AP-2 and AP180 affect the recruitment and/or the stability of clathrin at the contractile vacuole.

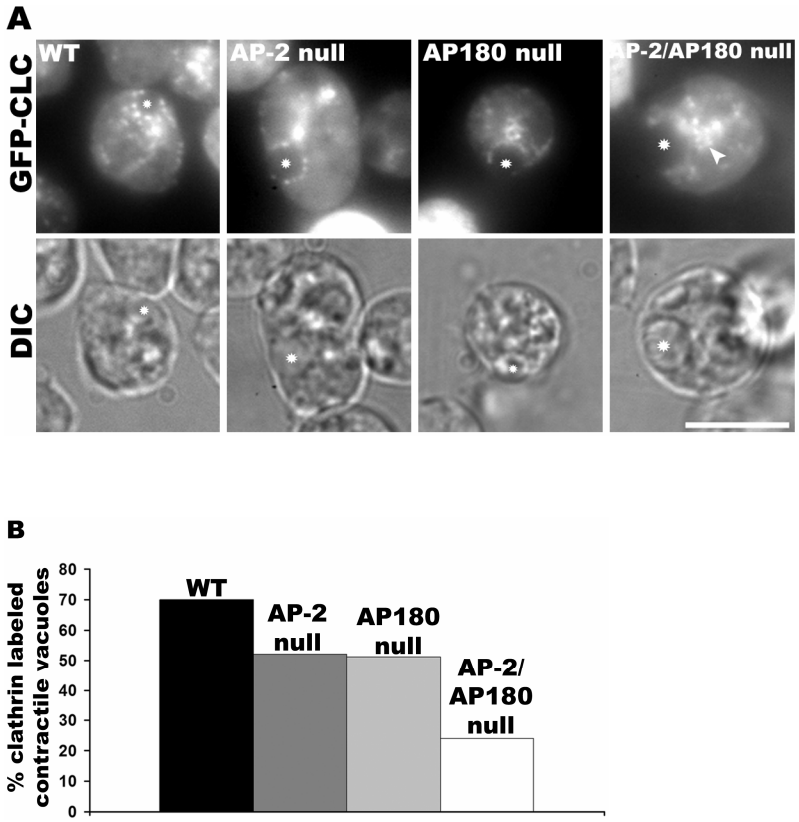


Figure 3.7: The association of clathrin at the contractile vacuole is reduced in AP-2a/AP180 double null mutants. (A). Living wild-type, AP-2 null, AP180 null and AP-2 /AP180 double null cells expressing GFP-clc in hypotonic media were imaged using fluorescent microscopy. In wild-type, AP-2 null and AP180 null cells, GFP-clc punctae decorated the bladder and sometimes the tubules of the contractile vacuole. However, in AP-2 /AP180 null cells this association of clathrin with the contractile vacuole was usually replaced by a cluster of GFP-clc punctae near the expanded bladder. (B). Clathrin

labeled contractile vacuoles in wild-type (n=64), AP-2 (n=73), AP180 (n=47) and AP-2 /AP180 double null (n=74) cells were quantified. Scale bar 10μm.

3.3 DISCUSSION

In this study we found that AP-2 requires AP180 to form stable clusters on the plasma membrane and that both adaptor proteins are required for 30% of the clathrin association on the plasma membrane. These results suggest that AP180 and AP-2 co-operate to recruit a portion of clathrin on the plasma membrane and that other protein and/or lipid interactions target clathrin to the plasma membrane in the absence of AP180 and AP-2. Additionally, AP-2 and AP180 function redundantly to recruit clathrin on the contractile vacuole and are both involved in the regulation of contractile vacuole size in both nutrient media and water.

3.3.1 AP-2 and AP180 cluster at the same sited on the plasma membrane but do not require each other to localize clathrin

AP-2 and AP180 have been shown to bind in vitro and to colocalize extensively in HeLa cells (Hao *et al.*, 1999; Meyerholz *et al.*, 2005). Our results are in agreement with previous studies that mammalian AP180 and AP-2 colocalize on the plasma membrane (Meyerholz *et al.*, 2005) and we extend these studies by showing that both AP-2 and AP180 remain associated with clathrin in the absence of each adaptor. This is not surprising since both proteins can directly bind to clathrin (Keen and Beck, 1989; Gallusser and Kirchhausen, 1993; Ye and Lafer, 1995b; Ford *et al.*, 2001). However, we found that in the absence of AP180, the number of AP-2 punctae on the plasma membrane was reduced while the number of bright cytoplasmic punctae increased. This would suggest that without AP180, AP-2 cannot stably associate with the plasma membrane. It is also possible that the increase in cytoplasmic AP-2 punctae is a result of

the inability of AP-2 to be directed to the plasma membrane without AP180 and AP-2 inappropriately assembles into clathrin containing punctae in AP180 null cells. However, we don't favor this possibility because AP-2 can be directed to the plasma membrane via its interaction with phosphodityl inositides independently of AP180. Therefore, it is more likely that AP-2 recognizes the target proteins and binds phosphodityl inositides on the plasma membrane independently of AP180. However, without AP180 to stabilize the growing clathrin lattice it dissociates from the plasma membrane. While AP180 appears to stabilize the association of AP-2 with the membrane, the reverse is not true. This suggests that AP180 binding to clathrin and phospholipids is sufficient for its stable association with the plasma membrane in *Dictyostelium*. In contrast, an RNA knockdown of AP-2 in HeLa cells decreased the association of AP180/CALM with the plasma membrane suggesting that the dependence of adaptor proteins on each other may differ in different systems (Meyerholz *et al.*, 2005).

3.3.2 AP-2 and AP180 influence clathrin assembly on the plasma membrane

AP-2 and AP180 are considered the two main clathrin assembly proteins at the plasma membrane. In vitro, these proteins act synergistically in clathrin assembly (Hao *et al.*, 1999). In this study we examined whether the absence of both adaptors would abolish the membrane association of clathrin. Indeed we found that the association of clathrin on the plasma membrane was decreased when both AP-2 and AP180 were absent. We noticed some decrease in clathrin membrane association in the single AP-2 and AP180 null cells. However, in the double null cells, the total decrease in clathrin assembly on the plasma membrane was less than the sum of the decrease in the single mutants. This result suggests that AP-2 and AP180 assembly clathrin in a co-operative and redundant manner rather than synergistically. It is likely other adaptors or endocytic proteins also contribute to this function. Indeed, both AP-2 and AP180 contain domains that bind to other

endocytic proteins such as Eps15 that can link clathrin to the plasma membrane. Preliminary studies in another cells line showed that deletion of AP-2 from Ax2 cells diminished 40% of plasma membrane-associated clathrin, whereas plasma membrane associated clathrin was diminished by only 17% in AP-2 deleted in DH1 cells. The discrepancy might be explained by the difference in parental cell lines from which the two mutant strains were developed. Conceivable the deletion of both AP-2 and AP180 genes in the Ax2 background would severely affect the membrane association of clathrin. Still unresolved is whether the remaining 60% of clathrin on the plasma membrane in the AP-2/AP180 double null cells is functional or whether it assembles into morphologically regular cages.

3.3.3 AP-2 and AP180 are required for normal contractile function

While endocytic compartments do not normally overlap with the contractile vacuole system, these membrane systems may merge in stressed cells. Heuser suggested that clathrin normally is not involved in the recycling of contractile vacuole membrane following its collapse onto the plasma membrane (Heuser, 2006). However, if *Dictyostelium* cells are treated with lantrunculin or plated on polylysine coverslips, harsh treatments that stress cells, clathrin is seen frequently near the collapsed contractile vacuole presumably retrieving membrane and proteins required to regenerate the contractile vacuole (Heuser, 2006). Given the frequency at which we see clathrin on the contractile vacuole in wild-type cells, clathrin might play a more direct role at the contractile vacuole than was originally thought. The decreased association of clathrin on the contractile vacuole in the single AP180 and AP-2 null cells also indicates that efficient assembly of clathrin on the contractile vacuole requires these two adaptor proteins. We found that AP-2 localized on the contractile vacuole although rarely. When AP-2 was found on the contractile vacuole it was organized as punctae associated with

AP180. Two other studies have shown that the μ-subunit of AP-2 binds to and is required for the activity of the V-H^+ATPase from clathrin coated vesicles in bovine brain extracts (Myers and Forgac, 1993; Liu *et al.*, 1994). These results suggest that AP-2 could also bind the V-H^+ATPase on the contractile vacuole and affect contractile vacuole function in *Dictyostelium*. Deletion of both AP-2 and AP180 substantially affected the localization of clathrin on the contractile vacuole. The clustering of clathrin punctae to one side of the contractile vacuole suggested that clathrin could not be properly tethered to the membrane of the bladder but possibly remained associated with residual tubular structures. Given that the decrease in clathrin association on the contractile vacuole is greater in the double null cells compared to the single null cells, it seems likely that AP180 and AP-2 function redundantly to promote clathrin assembly on the contractile vacuole. Furthermore, deletion of AP-2 and AP180, both proteins that function in endocytosis on the plasma membrane, renders the cells osmosensitive. Therefore, we propose that following the discharge of the contractile vacuole, AP-2 and AP180 function with clathrin to retrieve contractile vacuole membrane and proteins that have been inappropriately deposited on the plasma membrane when the contractile vacuole fused. On example could be contractile vacuole v-SNAREs that could be required for the proper fusion of the contractile vacuole with the plasma membrane. In the absence of these adaptor proteins, clathrin is inefficiently assembled on the plasma membrane and the contractile vacuole therefore coated pits fail to form and to recycle back proteins to the contractile vacuole. This scenario explains why enlarged contractile vacuoles form and linger near the plasma membrane in AP-2 /AP180 double null cells; these contractile vacuoles fail to fuse because they are deficient in proteins important for fusion.

In summary, the colocalization images and the osmoregulation analysis of the AP-2 /AP180 double null cells are in agreement with previously reported data that AP-2 and

AP180 function together. Each adaptor protein affects the association of clathrin with the plasma membrane and AP180 is required for the stable association of AP-2 with the plasma membrane. We also find that both assembly proteins contribute to clathrin localization on the contractile vacuole in *Dictyostelium* cells, and that AP180 is required for the stable association of AP-2 with the plasma membrane. Taken together, our study provides new evidence that these two endocytic proteins are involved in the contractile vacuole function and clathrin assembly on the contractile vacuole.

Chapter 4: Functional domain analysis of AP180

4.1 INTRODUCTION

All members of the AP180 family share similar domain organization. They have a structurally conserved AP180 NH2-terminal homology domain (ANTH) at their NH_2 terminus (Legendre-Guillemin *et al.*, 2004). This domain recognizes and binds to phosphoinositides at the plasma membrane such as Ptd(4,5)P_2, and is found in many proteins of the endocytic machinery (Ford *et al.*, 2001). Until recently this structural module was named ENTH domain for Epsin NH2-terminal homology domain because the two modules are structurally very similar to one another (De Camilli *et al.*, 2002). The ANTH domain is present in various other endocytic proteins outside the AP180 family such as HIP1 (Huntingtin Interacting Protein) and the related protein, HIP1R, which functions as a link between the actin cytoskeleton and clathrin (Engqvist-Goldstein *et al.*, 1999; Mishra *et al.*, 2001). AP180 binds to phosphoinositides and preferentially to PIP2 (Norris *et al.*, 1995; Ye *et al.*, 1995; Hao *et al.*, 1997; Mao *et al.*, 2001). Binding of the ANTH domain to PIP2 occurs through a conserved lysine rich region defined as (K/G)A(T/I)x6(P/L/V)KxK(H/Y)(Ford *et al.*, 2001), a sequence absent from the ENTH domain (Ford *et al.*, 2001). While binding of ENTH-domain containing proteins such as Epsin, to PIP2 at the membrane induces curvature of a lipid monolayer, AP180 does not induce curvature (Ford *et al.*, 2002; Stahelin *et al.*, 2003). This points out to important mechanistic differences between different proteins of the endocytic machinery.

Members of the AP180 family also share a clathrin binding domain with the consensus sequence L(L,I)(D,E,N)(L,F)(D,E) (Dell'Angelica *et al.*, 1998; Kirchhausen, 2000). This domain is responsible for binding to the -propeller N-terminal domain of

clathrin heavy chain (ter Haar *et al.*, 2000). In addition, a DLL motif, mediates binding of AP180 proteins to clathrin (Morgan *et al.*, 2000). The *Dictyostelium* AP180 lacks a DLL motif whereas the *A. thaliana, Drosophila*, *C. elegans* and the mammalian AP180 homologues contain one or more DLL motifs (Zhang *et al.*, 1998; Nonet *et al.*, 1999; Morgan *et al.*, 2000; Barth and Holstein, 2004). AP180 orthologs also contain a DPW/DPF motif which has been shown to confer binding to the -hinge region of AP2 (Owen *et al.*, 1999). Direct binding between AP-2 and AP180 has been shown in vitro and accounts for their cooperative clathrin assembly activity (Hao *et al.*, 1999). Furthermore, members of the AP180 family also contain one or more NPF motifs which confers binding to Eps15 homology (EH) containing proteins (Salcini *et al.*, 1999; Confalonieri and Di Fiore, 2002). One such protein is the yeast Pan1p, a homologue of the mammalian endocytic protein Eps15, that binds the actin cytoskeleton and the yeast AP180 (Wendland and Emr, 1998). The domain organization of AP180 is a reflection of the multiplicity in protein interaction that is required to drive the formation of a clathrin coated vesicle.

Most of what we know about the function of the different domains in AP180 comes from in vitro studies. A recombinant fragment from mouse AP180 that contains the AP-2 binding site inhibits the interaction between AP180 and AP-2 and their cooperative clathrin assembly activity (Hao *et al.*, 1999). Also, mutations in the AP-2 binding motifs of the non-neuronal AP180 homologue, CALM, reduces its binding to AP-2 by up to 70% (Meyerholz *et al.*, 2005). Furthermore, the 33kDa amino terminus of bovine AP180 has a high affinity for inositol polyphosphates ($InsP_6$) however this binding inhibits clathrin assembly (Norris *et al.*, 1995; Ye *et al.*, 1995). More recently it was found that the *Arabidopsis* DLL motif in AP180 is required for the clathrin assembly activity of AP180 but it is not necessary for binding of AP180 to clathrin (Barth and

Holstein, 2004). Recently it was shown that expression of the clathrin-binding domain of AP180 inhibits endocytosis of transferrin in mammalian cells by mislocalizing clathrin away from endocytic sites (Zhao *et al.*, 2001).

In this study, I have made two truncated *Dictyostelium* AP180 constructs, $AP180_{1-147}$ and $AP180_{239-695}$, tagged with GFP and using site-directed mutagenesis I mutated two of the three amino acids in the AP-2 and EH-domain binding motifs . Our results show that GFP- C-terminus was toxic to the cells and therefore expression was poor. In rare cases where cells expressed the GFP- C-terminus construct, the truncated proteins localized exclusively to the nucleus. We also found that the ANTH domain is not necessary for the localization of AP180 and clustering with clathrin but it is required for the function of AP180 in osmoregulation. Furthermore, we show that both AP-2 and EH-domain binding motifs are dispensable for the localization of AP180 at the plasma membrane.

4.2 RESULTS

4.2.1 The ANTH domain of AP180 localizes to the nucleus

To test whether the ANTH domain is necessary for the localization of AP180 on the plasma membrane, I generated a truncated mutant, $AP180_{1-147}$, that included the conserved amino acid sequence of the ANTH domain (residues 28-41) (Figure 4.1). This construct was tagged with GFP at the amino terminus (M.W.=42kDa) and transformed into wild-type and AP180 null cells. Both cell strains showed that the truncated protein was expressed at lower amounts than GFP alone (25kDa) (Figure 4.2).

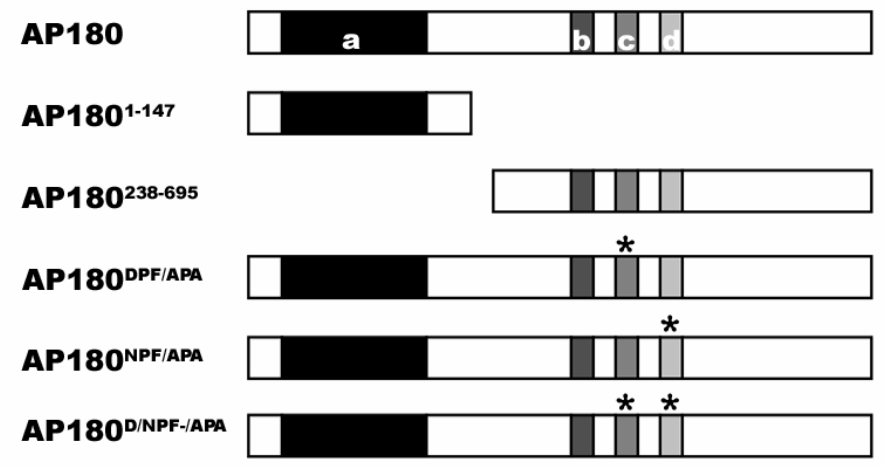


Figure 4.1: Domain analysis of AP180. Two truncated and three mutated constructs of AP180 used in this study. Asterisks denote sites of mutagenesis (DPF and NPF motifs were mutated to APA); a:ANTH, b: clathrin box, c: DPF, d: NPF.

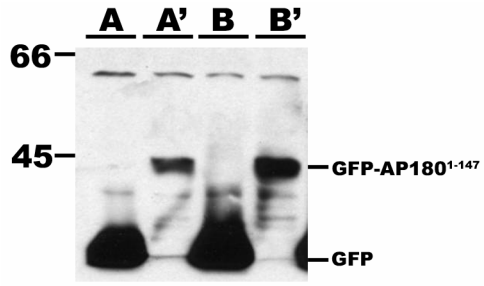


Figure 4.2: Expression levels of GFP-AP180$_{1-147}$ and GFP vector in wild-type and AP180 null cells. Western blot stained with an antibody against GFP. Cells lines were A: GFP vector alone in wild-type cells (Ax2); A': GFP-AP180$_{1-147}$ in wild-type; B: GFP vector alone in AP180 null cells; B': GFP-AP180$_{1-147}$ in AP180 null cells (1x10^6 cells/lane).

Cells expressing GFP-AP180$_{1-147}$ fixed and stained with 4'-6-Diamidino-2-phenylindole (DAPI) showed that GFP-AP180$_{1-147}$ localized exclusively to the nucleus whereas cells expressing the GFP vector only faintly labeled the nucleus (Figure 4.3). In contrast, GFP-ENTH$_{1-435}$ from the *Dictyostelium* epsin ortholog uniformly labeled the plasma membrane as well as the nucleus (data not shown). Because many cells did not express the construct, and because the expression level of GFP-AP180$_{1-147}$ was lower in than GFP in both wild-type and AP180 null cells, we concluded that this construct was toxic to the cells.

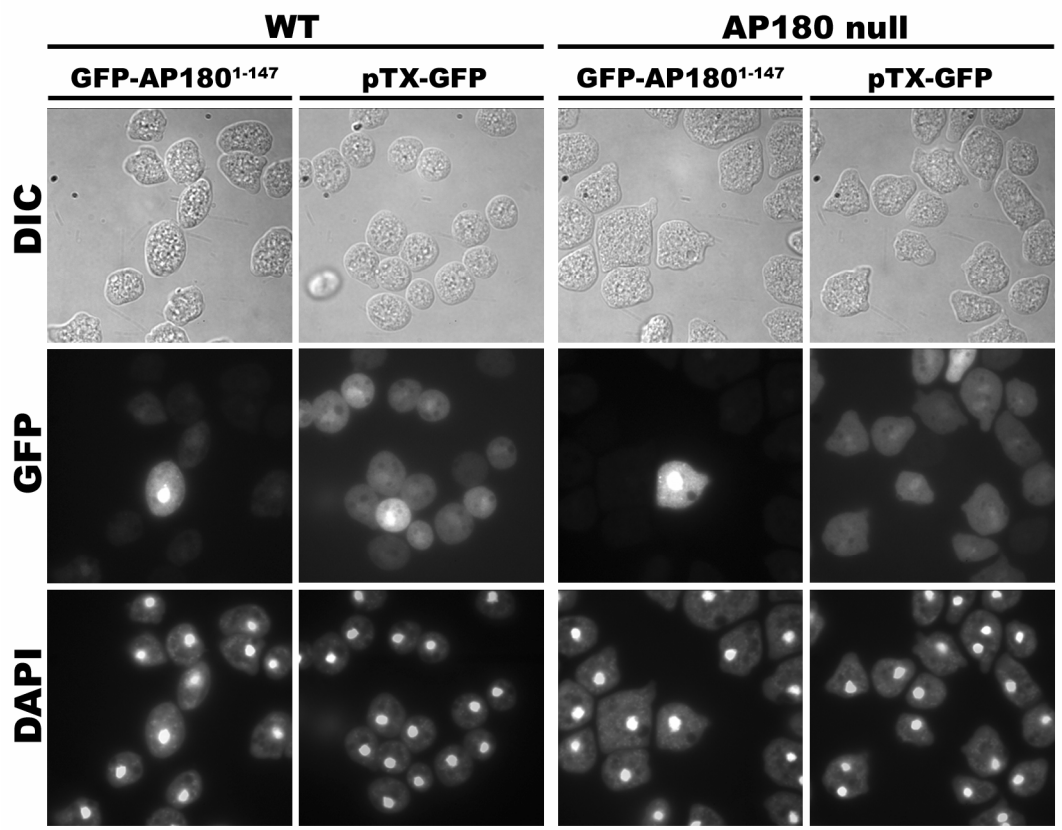


Figure 4.3: The N-terminal ANTH domain localizes to the nucleus. Wild-type (Ax2) and AP180 null cells expressing GFP-AP180$_{1-147}$ or just the pTX-GFP vector were fixed and

stained with DAPI to show nuclear localization. Note that only a few cells expressed the truncated construct. Scale bar, 10μm

4.2.2 The ANTH domain of AP180 is not required for the localization of AP180 on the plasma membrane and colocalization with clathrin.

The AP180 C-terminus includes the clathrin box, AP-2 and EH-domain binding motifs. To investigate the properties of this domain, we imaged cells expressing GFP-tagged $AP180_{239-695}$ (M.W.=76kDa). Both wild-type and AP180 null cells showed similar results and I will only present findings from GFP-$AP180_{239-695}$ expressed in AP180 null cells (Figure 4.4). In wild-type and AP180 null cells, full length GFP-AP180 localized as punctae on the plasma membrane, on the contractile vacuole membrane and in the cytoplasm. Similarly, GFP-$AP180_{239-695}$ localized as punctae on the plasma membrane, on the contractile vacuole membrane and in the cytoplasm (Figure 4.4 top and bottom left panels). Thus, the absence of the amino terminal 238 residues that includes the ANTH domain did not affect the localization of AP180. Staining of GFP-AP180 expressing cells with an antibody against clathrin showed extensive colocalization between AP180 and clathrin on the plasma membrane and cytoplasm. GFP-$AP180_{239-695}$ expressing cells stained with a clathrin antibody showed that the C-terminal portion of AP180 was sufficient for colocalization with clathrin (Figure 4.4).

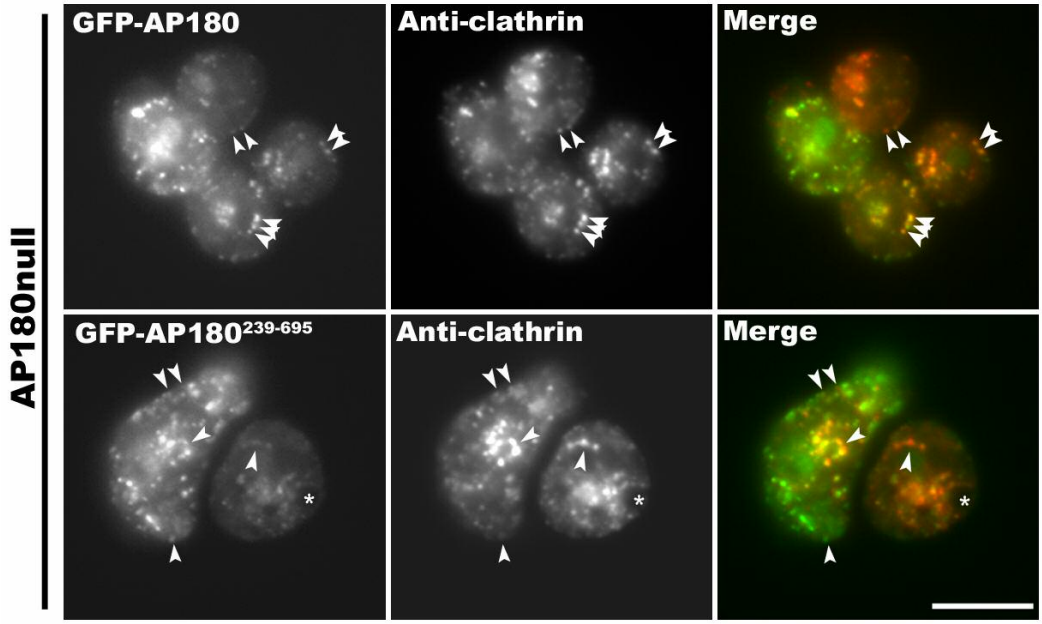


Figure 4.4: The ANTH domain is not required for normal localization of AP180. AP180 null cells expressing GFP-AP180 (top panels) or GFP-AP180$_{239-695}$ (bottom panels) were fixed and stained with an antibody against clathrin conjugated to Texas Red secondary antibody (middle panels). Both proteins colocalized with clathrin punctae on the plasma membrane and cytoplasm (arrows). GFP-AP180$_{239-695}$ and clathrin also clustered in the same punctae on the contractile vacuole (asterisks – bottom panel). Scale bar, 10μm.

4.2.3 The ANTH domain is required for AP180 function in osmoregulation

The localization of GFP-AP180$_{239-695}$ on the plasma membrane, contractile vacuole and cytoplasm suggested that GFP-AP180$_{239-695}$ also be functional. To examine whether GFP-AP180$_{239-695}$ could rescue the osmoregulation defect of AP180 null cells, wild-type cells in water were scored for the presence of large contractile vacuoles using DIC microscopy. In nutrient media, wild-type, AP180 null and GFP-AP180$_{239-695}$ expressing

cells displayed normal size contractile vacuoles. In water, wild-type cells displayed the same contractile vacuole morphology whereas AP180 null and GFP-AP180$_{239-695}$ expressing cells displayed abnormally large contractile vacuoles (Figure 4.5). This result suggests that the C-terminal portion of AP180 is not sufficient to restore function of AP180 in the regulation of contractile vacuole size and implicates the ANTH domain as a requirement for AP180 function.

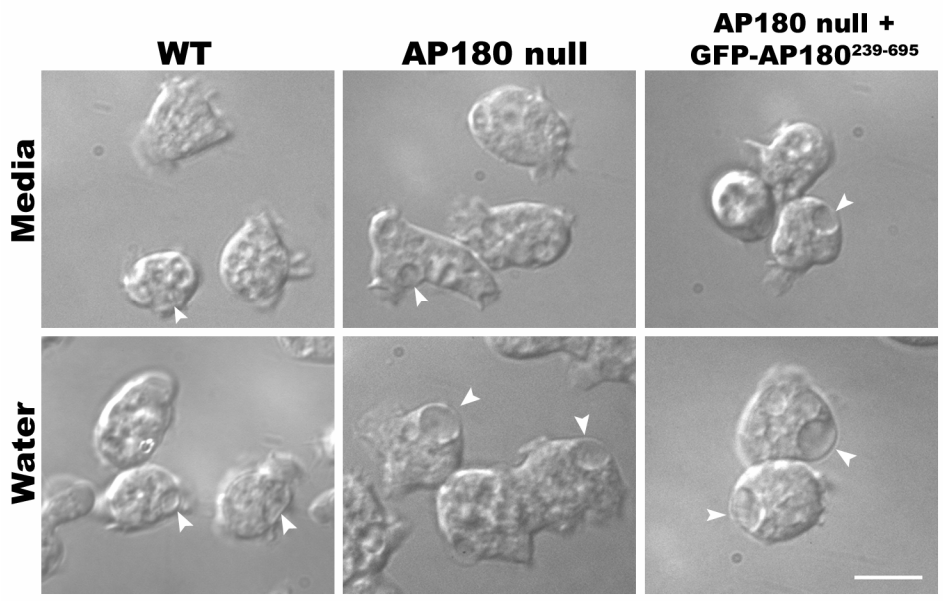


Figure 4.5: The C-terminus of AP180 does not rescue the osmoregulatory defect of AP180 null cells. Wild-type, AP180 null and AP180 null cells expressing GFP-AP180$_{239-695}$ were imaged using DIC microscopy. In media (top panels) all cell lines displayed normal size contractile vacuoles (arrows) whereas in water (bottom panels) AP180 null cells and AP180 null cells expressing GFP-AP180$_{239-695}$ showed enlarged contractile vacuoles. Scale bar, 10μm

4.2.4 Mutations in the AP-2 and EH-domain binding motifs do not affect the overall localization of AP180

Using site-directed mutagenesis, we generated three different constructs of mutated AP180. First, the AP-2 binding motif, DFF, was mutated to APA. Second, the EH-domain binding motif, NPF, was mutated to APA and finally both AP-2 and EH-domain binding motifs were mutated to APA in the same construct (Figure 4.1). These constructs were tagged with GFP at their N-terminus and expressed in wild-type and AP180 null cells (Figure 4.6). Imaging of fixed wild-type cells expressing the mutagenic constructs showed that GFP-AP180$^{DPF/APA}$, GFP-AP180$^{NPF/APA}$ and GFP-AP180$^{D/NPF/APA}$ localized as punctae on the plasma membrane, within the cytoplasm and in a perinuclear area. This localization was similar to that of GFP-AP180 expressed in wild-type cells. In AP180 null cells, GFP-AP180$^{DPF/APA}$, GFP-AP180$^{NPF/APA}$ and GFP-AP180$^{D/NPF/APA}$ also formed punctae on the plasma membrane, cytoplasm and perinuclear area. However, the cytoplasmic punctae formed by the mutated constructs were more abundant in AP180 null cells than in wild-type or AP180 null cells expressing GFP-AP180. This increase in cytoplasmic punctae was greater when both motifs were mutated than either motif alone. The presence of AP180$^{DPF/APA}$, GFP-AP180$^{NPF/APA}$ and GFP-AP180$^{D/NPF/APA}$ punctae on the plasma membrane suggests that these motifs are not required for the recruitment of AP180 to the plasma membrane. However, the association of AP180 with the plasma membrane might be compromised when the AP-2 binding and the EH-domain binding motifs are mutated. These results suggest that in the absence of endogenous AP180, the disruption of the AP-2 and EH-domain containing motifs affects the localization of AP180.

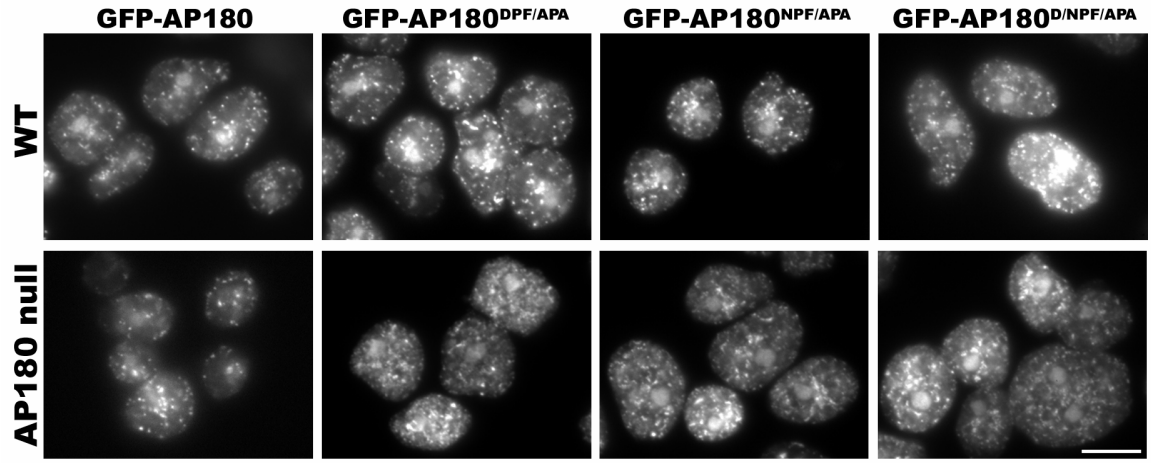


Figure 4.6: AP-2 and EH-domain binding motifs are not essential for punctate localization of AP180. Using site-directed mutagenesis the AP-2 binding motif (DPF) and the EH-domain binding motif (NPF) were mutated to APA. GFP-tagged constructs were transformed in wild-type (Ax2) and AP180 null cells and visualized using fluorescence microscopy. All constructs localized as punctae in the cytoplasm and on the plasma membrane in wild-type and AP180 null cells. Nuclear and perinuclear staining was also noticeable. Punctae formed by the GFP-AP180$^{DPF/APA}$, GFP-AP180$^{NPF/APA}$ and GFP-AP180$^{D/NPF/APA}$ constructs in AP180 null cells, were more abundant in the cytoplasm. Scale bar, 10μm

4.3 DISCUSSION

4.3.1 The ANTH domain of AP180 localizes to the nucleus

AP180 possesses specific sequence motifs that account for binding to phosphoinositides, clathrin and endocytic proteins thus serving as a link between clathrin and the endocytic machinery on the membrane. The N-terminal ANTH domain which is

found in all members of the AP180 family binds Ptd(4,5)P_2 on the plasma membrane and is required for the clathrin assembly activity of AP180 (Ford *et al.*, 2001; Holstein and Oliviusson, 2005). Recently, another intriguing characteristic of ENTH/ANTH domain containing proteins was highlighted. It was found that the ENTH domain of epsin binds a transcription factor in the nucleus and that shuttling of full length epsin in and out of the nucleus is inhibited by leptomycin B (an inhibitor of the CRM1-dependent pathway) (Hyman *et al.*, 2000). Furthermore, the non-neuronal homologue of AP180, CALM, and another clathrin accessory protein, Eps15, have also been shown to undergo nucleo-cytoplasmic shuttling, acting as transcriptional regulators in a GAL4-based activation assay (Vecchi *et al.*, 2001). Notably, CALM was first identified from a chromosomal translocation that resulted in fusion of CALM with the transcription factor AF10 (Dreyling *et al.*, 1996). We have noticed a prominent nuclear staining with both GFP-AP180 and GFP-$AP180_{1-147}$ therefore it is possible that the ANTH domain of *Dictyostelium* AP180 could be shuttling AP180 to the nucleus. Sequence analysis of the *Dictyostelium* AP180 did not reveal the presence of a canonical nuclear localization (NLS) or export signal (Nakielny and Dreyfuss, 1999). However, the ANTH domain and classical NLSs are both lysine rich sequences.

The absence of GFP-$AP180_{1-147}$ from the plasma membrane was surprising since the presence of the ANTH domain theoretically would target this construct to the plasma membrane via phospholipid interaction. However, the low expression of this construct and the slow growth rate of transformants observed suggest that the expressed GFP-$AP180_{1-147}$ was toxic to the cells.

4.3.2 The C-terminus of AP180 is sufficient for normal localization but not for function of AP180.

Direct binding of AP180 to clathrin, AP-2 and Eps15 has been demonstrated by in vitro assays (Dell'Angelica *et al.*, 1998; Owen *et al.*, 1999; Morgan *et al.*, 2003) and the domains responsible for binding to these proteins are found in the *Dictyostelium* AP180. The interaction of AP180 with other endocytic proteins like AP-2 and Eps15 is believed to be important in the formation of a stable platform where clathrin assembly can occur on the plasma membrane. The mutations introduced in the AP-2 and EH domain binding motifs of AP180 had no effect on the localization of AP180 when expressed in wild-type cells. This result suggests two things. First, that the AP-2 and EH-domain binding motifs are dispensable for the proper localization of AP180 or that the mutated constructs can be targeted to the correct site because they can associate with the endogenous wild-type AP180. We favor the latter possibility because in the absence of endogenous AP180 (AP180 null cells) there is an increase in cytoplasmic punctae formed by $AP180^{DPF/APA}$, GFP-$AP180^{NPF/APA}$ and GFP-$AP180^{D/NPF/APA}$. The increase in cytoplasmic punctae indicates that the AP-2 and/or the EH-domain binding motifs are important either for the stable association of AP180 on the plasma membrane or for specifying the sites for AP180 localization. It is likely that AP180 is targeted to sites specified by the presence of AP-2 and/or EH-domain containing proteins because AP180 can directly interact with these proteins. When the AP-2 and/or EH-domain binding motifs of AP180 are disrupted, AP180 has less affinity for the proper target sites, and its localization into punctae becomes more diffuse. Mutations in the DPF motif of AP180/CALM has been shown to decrease, but not completely abolish, binding of AP180 to AP-2 (Meyerholz *et al.*, 2005). The residual binding is thought to be through an interaction with EH-domain containing proteins like Eps15 which binds AP180 and AP-2. Indeed, our results suggest that when

both AP-2 and EH-domain binding motifs are mutated, the effect on the localization of AP180 is greater than when each motif is mutated alone.

The domain analysis of AP180 revealed that the ANTH domain is required for the function of AP180 in osmoregulation but not for proper localization of AP180. The presence of the clathrin binding domain, AP-2 and EH-domain binding motifs are sufficient to localize AP180 punctae on the plasma membrane and cytoplasm. The full effect of the disruption of the AP-2 and/or the EH-domain binding motif of AP180 and how it affects the clustering of AP180 with clathrin still needs to be determined.

Chapter 5: Conclusions and future directions

AP180, one of many proteins involved in clathrin-mediated endocytosis, stimulates the assembly of clathrin lattices on membranes, but its unique contribution to clathrin function remains elusive. In this study, the *Dictyostelium discoideum* ortholog of the adaptor protein AP180 was identified and a mutant strain carrying a deletion in this gene was characterized. The role of AP180 in clathrin mediated processes in *Dictyostelium discoideum* was assessed. I concluded that AP180 and clathrin function in the regulation of contractile vacuole size and activity. A strain carrying a deletion in both AP-2 and AP180 genes was also constructed and phenotypic characterization of this mutant strain revealed that two clathrin adaptor proteins function in osmoregulation. This study raises interesting questions about the role of clathrin-mediated processes and clathrin adaptor proteins such as AP180 during osmoregulation.

5.1 THE ROLE OF AP180 DURING OSMOREGULATION

5.1.1 Finding binding partners of AP180 at the contractile vacuole

This study found that a clathrin adaptor protein localizes to the contractile vacuole and has a direct role in the regulation of contractile vacuole size. It also strengthens previous studies that have highlighted the importance of clathrin in osmoregulation by providing strong evidence of clathrin localization at the contractile vacuole. One important question that arises from this work is whether clathrin and AP180 function to retrieve proteins from the contractile vacuole membrane. What type of proteins on the contractile vacuole could be targets of AP180? One way to answer this would be to find binding partners of AP180 using in vitro assays. A construct of AP180 tagged with Tap-

tag has already been made although the functionality of this construct has not been tested yet. If found to be functional and can rescue the osmoregulatory defect of AP180 null cells it can be used to pull down potential binding partners from whole cell lysates. Any binding partners identified by either western blot analysis or mass spectroscopy could be examined for their cellular localization and their role in osmoregulation. Recently a family of twelve genes encoding SNAREs (N-ethylmaleimide-sensitive fusion protein attachment protein receptors) was identified in *Paramecium tetraurelia* and one out of the four synaptobrevins (a subclass of SNARE proteins) examined (PtSyb2) was localized to the contractile vacuole (Schilde *et al.*, 2006). SNAREs are very likely to function at the contractile vacuole in *Dictyostelium discoideum* because they are key players in all membrane fusion events of the endocytic pathway (Jahn and Scheller, 2006). SNAREs at the contractile vacuole would be involved in the fusion of the contractile vacuole with the plasma membrane at the point of expulsion. It is known that during fusion of the contractile vacuole bladder with the plasma membrane there is minimal mixing between components of the contractile vacuole and the plasma membrane. The minimal mixing of contractile vacuole proteins could be the result of their efficient retrieval from the plasma membrane by clathrin coated pits. Evidence to support this hypothesis comes from the presence of clathrin punctae when the contractile vacuole discharges. A role for AP180 in retrieving v-SNAREs from the plasma membrane is intriguing; the v-SNARE synaptobrevin in *C. elegans* and *D. melanogaster* is mislocalized to the extrasynaptic azonal region in AP180 mutants (Nonet *et al.*, 1999; Bao *et al.*, 2005). Whether AP180 binds directly to synaptobrevin is yet to be determined but the effect on SNARE localization in the absence of AP180 suggests that AP180 not only assembles clathrin but also is involved in retrieving the fusion machinery from the plasma membrane. Homologues of synaptobrevin in *Dictyostelium* exist, albeit with low sequence

homology, therefore it would be interesting to find out whether these homologues have any function at the contractile vacuole and if they are targets of AP180.

5.1.2 Clathrin assembly at the contractile vacuole in the absence of AP180

In other organisms, AP180 is known to be responsible for assembly of clathrin into cages of homogeneous size (Zhang *et al.*, 1998; Nonet *et al.*, 1999; Barth and Holstein, 2004; Meyerholz *et al.*, 2005). Naturally, a question that still remains unanswered is whether clathrin lattices formed at the contractile vacuole in the absence of AP180 have an abnormal size or structure. The morphology of clathrin cages in wild-type and AP180 mutant cells can be examined using electron microscopy to determine whether AP180 in *Dictyostelium* is responsible for the assembly of clathrin into cages of uniform size and shape as in other systems. Furthermore, electron microscopy of wildtype and AP180 null *Dictyostelium* cells that have been exposed to hyposmotic stress could reveal major differences in clathrin coat assembly especially at the contractile vacuole. An inefficient assembly of clathrin in the absence of AP180 could also account for the enlarged contractile vacuoles seen in these mutant cells.

5.1.3 Dissecting the relationship between AP180 and the contractile vacuole.

Mutants with known osmoregulatory defects such as the clathrin light chain and clathrin heavy chain null cells have been used in this study to examine the localization of AP180. Indeed, we found that mutants with disrupted contractile vacuoles also have differences in AP180 localization. To extend these experiments, we could examine the effect on the localization of AP180 when the contractile vacuole network is pharmacologically disrupted. Concanamycin A has been shown to inhibit the activity of V-H^+ATPase and consequently disrupt the activity of the contractile vacuole (Temesvari *et al.*, 1996). Cells treated with concanamycin A adapt after an hour in the presence of

the drug in nutrient media (Temesvari *et al.*, 1996), therefore the localization of AP180 at the contractile vacuole can be examined immediately after drug treatment and following adaptation to the drug. The effect on the localization of AP180 when the contractile vacuole is disrupted would provide further evidence of the involvement of AP180 in osmoregulation.

Clathrin heavy chain and clathrin light chain mutants are both osmosensitive. Clathrin heavy chain null cells completely lack a functional contractile vacuole network, whereas clathrin light chain null cells show less severe defects in contractile vacuole organization and less severe osmosensitivity. Conceivably, AP180 and clathrin light chain could function together in osmoregulation. To explore this possibility, an AP180/clathrin light chain double null cell strain could be constructed and used to examine the contractile vacuole dynamics and morphology. It is possible that in the absence of both AP180 and clathrin light chain the size of the contractile vacuole would be severely affected, fusion of the contractile vacuole with the plasma membrane inhibited or generation of a functional contractile vacuole could be compromised. The combined effect of both proteins on the contractile vacuole activity would provide further evidence of a functional relationship between AP180, clathrin light chain and the contractile vacuole.

5.2 DOES AP180 FUNCTION AT THE GOLGI COMPLEX?

GFP tagged AP180 localized to the perinuclear area where it also colocalized extensively with clathrin. This result is in agreement with studies in mammalian cells where AP180/CALM was shown to colocalize with clathrin in the Golgi area (Tebar *et al.*, 1999). As in other model systems, clathrin is believed to function at the Golgi in *Dictyostelium* cells (Lefkir *et al.*, 2003) therefore an aspect of AP180 function that still

remains unexplored is whether it functions with clathrin at the Golgi, and if so what its role is at this site. We have shown that in the absence of AP-1, the Golgi associated clathrin assembly protein, AP180 localizes almost exclusively to the plasma membrane. Additionally, it was found that purified bovine AP-1 binds AP180 which suggests a possible relationship between the two proteins in vivo (Hao *et al.*, 1999). Furthermore, it has been shown that depletion of AP180/CALM in HeLa cells redistributes AP-1 from the Golgi to cytoplasmic punctate structures (Meyerholz *et al.*, 2005). Therefore, there is evidence to suggest that AP180 functions with clathrin and AP-1 at the Golgi complex. Several different approaches can be taken to further explore the role of AP180 at the Golgi apparatus. First, to confirm the localization of AP180 at the Golgi complex in *Dictyostelium*, a Golgi marker like wheat germ agglutinin (WGA) can be used to stain cells expressing GFP-AP180. Colocalization between a specific Golgi marker and AP180 would provide strong evidence that the prominent perinuclear labeling we see in cells expressing GFP-AP180 represents the Golgi complex. Second, the effect of Golgi associated proteins like Golvesin, AP-1 and VwkA (Schneider *et al.*, 2000; Lefkir *et al.*, 2003; Betapudi *et al.*, 2005) could be examined in wildtype and AP180 null cells. Mislocalization of any of these proteins would indicate a relationship between AP180 and Golgi associated proteins and provide evidence that AP180 functions at the Golgi complex. Lastly, sorting events from the Golgi to other compartments of the endocytic pathway could be examined in AP180 null cells and compared to wildtype cells. For example, the precursors of the lysosomal enzymes -mannosidase and -glucosidase are transported from the endoplasmic reticulum to the Golgi and finally to lysosomes where they are proteolytically cleaved into the mature form (Cardelli *et al.*, 1986; Ruscetti *et al.*, 1994). Like clathrin, AP180 could be required for the sorting of -mannosidase and/or -glucosidase from the Golgi to lysosomes (Ruscetti *et al.*, 1994). To examine this

possibility, wildtype and AP180 null cells could be assayed for the -mannosidase and -glucosidase activity and secretion rates. Results from such assays would provide further insight into the function of AP180 at the Golgi.

5.2.1 Is there a link between AP180 at the Golgi and the contractile vacuole?

One very intriguing question that this study raises is whether AP180 is involved in sorting events from the Golgi to the contractile vacuole. It is known that the contractile vacuole network is derived from the Golgi apparatus (Gabriel *et al.*, 1999) and mutants like AP-1μ null cells are osmosensitive (even though AP-1 does not localize to the contractile vacuole). Furthermore, clathrin that localizes to the Golgi is also involved in osmoregulation. It is therefore plausible to suggest that clathrin mediated trafficking events provide a link between the Golgi complex and the contractile vacuole. AP180 could potentially be a key player in this process too. The ability of AP180 to bind to clathrin and AP-1, localize to the Golgi and the contractile vacuole suggests that it might also be acting as a clathrin adaptor protein in clathrin mediated sorting events from the Golgi to the contractile vacuole.

5.3 WHAT IS THE RELATIONSHIP BETWEEN AP180 AND OTHER ENDOCYTIC PROTEINS?

Epsin and Hip1R are part of the endocytic machinery that functions with clathrin (Chen *et al.*, 1998; Engqvist-Goldstein *et al.*, 2001). We have examined the localization of AP180 in the *Dictyostelium* Epsin null and Hip1R null cells and found that it localizes to plasma membrane and cytoplasmic punctae just like in wild-type cells. A closer examination of these cells to examine whether AP180 and clathrin still cluster in the same punctae without these two proteins might provide more information about the role

of AP180 on the plasma membrane. The reciprocal experiment where the localization of Epsin and Hip1R tagged with GFP is examined in the absence of AP180 could provide further insight as to how the localization of these accessory proteins is affected. AP-2 and AP180 colocalize in *Dictyostelium* wild-type cells and clathrin heavy chain null cells. Therefore it would be interesting to see whether this colocalization is perturbed in the absence of both Epsin and Hip1R (a double knockout of these two proteins has already been constructed in our lab). Similarly, clathrin and AP180 colocalize extensively in wild-type cells and AP-2 null cells but this colocalization could be altered by the absence of both Epsin and Hip1R. Additionally, a quantitative and qualitative analysis of the localization of AP180 at the contractile vacuole in the Hip1R/Epsin double knockout would provide additional insight into the interplay between endocytic proteins at the plasma membrane and the dynamics of AP180.

Phosphorylation plays an important role in clathrin coat formation (Brodsky *et al.*, 2001). Clathrin triskelia cannot assemble at physiological pH in the presence of clathrin light chain (Keen *et al.*, 1987; Chu *et al.*, 1999). However this inhibition is relieved by adaptor proteins like AP-2 through de-phosphorylation of the -chain hinge region (Wilde and Brodsky, 1996). There is evidence that AP180 is phosphorylated (Murphy *et al.*, 1994) and that phosphorylation of AP180 affects the combined clathrin assembly ability of AP180 and AP-2 (Hao *et al.*, 1999). The presence of a band that runs higher than the main AP180 band on western blots has been detected in *Dictyostelium* cell lysates. This suggests that *Dictyostelium* AP180 is also phosphorylated. This "double" band is still present in *Dictyostelium* AP-2 mutants (data not shown). To examine whether AP180 in *Dictyostelium* is phosphorylated a phosphatase assay could be performed. If the presence of a phosphorylated AP180 moiety is confirmed, then the phosphorylation state of AP180 could be examined in mutant cell lines such as Epsin, Hip1R, clathrin heavy chain and

clathrin light chain null cells. Results from these experiments will provide further information about the functional relationship between AP180 and other endocytic proteins at the plasma membrane.

5.4 THE IMPORTANCE OF AP180 IN THE ENDOCYTIC PATHWAY

5.4.1 The role of AP180 during endocytosis

Driven by adaptor proteins like AP-2 and AP180, clathrin assembly on the plasma membrane is required for the formation of a coated pit and endocytosis of selected cargo. The role of AP180 at the plasma membrane in *Dictyostelium* is still unclear. Clathrin and AP180 colocalize extensively at the plasma membrane, however AP180 does not seem to be involved in fluid phase uptake, a process that requires clathrin (O'Halloran and Anderson, 1992b). Therefore the question remains as to what is the role of AP180 at the plasma membrane. The endocytic pathway in *Dictyostelium* has been well studied and different compartments along the pathway have been identified using several markers and dyes. Our preliminary screen for defects in the endocytic pathway in AP180 null cells focused on the formation of lysosomal and post-lysosomal compartments using a Lysosensor dye and an antibody against vacuolin which localizes to post-lysosomes. Even though we found no difference in the number and morphology of lysosomes or post-lysosomes in AP180 null cells compared to wildtype cells, the number of post-lysosomal compartments increased in cells overexpressing GFP-AP180. This result could be due to the overexpression of AP180 or an artifact of overexpressing the tagged protein. To test this, AP180 can be cloned into a high-copy vector without the GFP tag. Cells overexpressing AP180 could be examined for an increase in post-lysosomal compartments. Furthermore, GFP-tagged vacuolin could be transformed in wildtype,

AP180 null and AP180 overexpressing cell strains to confirm the increase in number of post-lysosomal compartments seen with the anti-vacuolin antibody.

5.4.2 The role of AP180 during phagocytosis

Dictyostelium cells are avid phagocytes which makes them an excellent model system to study the dynamics of phagocytosis. Phagocytosis in *Dictyostelium* is an actin-dependent clathrin-independent process however key regulators of endocytosis like PIP2 also play an important role in phagocytosis (Seastone *et al.*, 1999; Lefkir *et al.*, 2004). Furthermore, the clathrin adaptor protein, AP-1 is also thought to be involved in phagocytosis (Lefkir *et al.*, 2004). AP180 null cells grow on bacterial lawns, conditions that require phagocytosis, at the same rate as wild-type cells, suggesting that AP180 is not required for phagocytosis. To examine further whether AP180 functions in phagocytosis, two additional experiments could be performed. First, GFP-AP180 expressing cells could be incubated with labeled yeast cells, fixed and visualized using fluorescence microscopy to determine if AP180 is found on the phagocytic cup. Second, GFP-AP180 expressing cells could be incubated with unlabelled yeast particles for an extended period of time (at least 1 hour), then fixed and stained with an antibody against p180 that localizes to late phagosomes and observe if there is colocalization between AP180 and p80. Additionally, the rate of phagosome maturation in wildtype and AP180 null cells could be tracked using fluorescently labeled bacteria and cells that have been pre-incubated in medium containing neutral red (Clarke and Maddera, 2006).

The future directions outlined in this section and the experimental approach can lead to a better understanding about the role of AP180 in *Dictyostelium* as well as in higher organisms. Elucidating the role of AP180 and clathrin at the contractile vacuole might help to better understand the functional relationship between AP180 and clathrin in neuronal cells of organisms such as *Drosophila* and *C. elegans*. Finding binding partners

of AP180 in *Dictyostelium* could point out homologues in other organisms that also function with AP180. Using this information would give us further insight as to how such protein-protein interactions facilitate the function of AP180. Examining the role of AP180 at the Golgi would enhance our knowledge on clathrin-mediated sorting events of lysosomal enzymes and other important proteins that are conserved in other model organisms. Finally, the information we receive from the study of AP180 in *Dictyostelium* would undoubtedly be a piece to the puzzle of clathrin-mediated endocytosis.

Chapter 6: Experimental Procedures

6.1 Materials and Methods

6.1.1 Cloning of *Dictyostelium* AP180

The *clmA* gene encoding the AP180 gene product was identified from a *Dictyostelium* genome database (www.dictybase.org) using a BLAST search (tBLASTn) with the first 300 amino acids of the mammalian neuronal AP180. Predicted protein domains at GeneDB identified an ANTH domain in the first 300 amino acids. Alignment and analysis of the predicted *Dictyostelium* AP180 protein sequence with protein sequences from other members of the AP180 family were performed using the Megalign program (DNAStar, Inc., Madison, WI). The percent identity between the *Dictyostelium* AP180 and those of other species was determined using the ClustalV parameters. A cDNA for the *clmA* gene was amplified using the Polymerase Chain Reaction (PCR) with primers selected from the genomic sequence (DDB0218102), 5´GGATCCATGTCGA CACCATGGGGAAAAGC3´ and 5´CCCGGGCTCGAGTATTTAAAAGTAAATATT TTGAACCTTTTGTTGTTG3´. The 2.1 kb amplified product was sub-cloned into the pTX-GFP expression vector (Levi *et al.*, 2000) at the BamHI and XhoI sites. This plasmid, pTX-GFP-AP180, was then introduced into cells by electroporation and transformants were selected in HL-5 medium supplemented with 10μg/ml G418 (geniticin; GIBCO BRL, Grand Island, NY).

6.1.2 Protein expression and generation of AP180 polyclonal antibody

The amplified AP180 cDNA was subcloned into the glutathione-S-transferase bacterial expression vector pGEX-2T (Smith and Johnson, 1988) using the BamHI and SmaI sites. GST-AP180 was transformed into *E. coli* BL-21 cells and the expressed

protein was purified from bacteria lysates as previously described (O'Halloran and Anderson, 1992a). The purified protein was used to raise rabbit polyclonal antisera against AP180 (Cocalico Biologicals; Reamstown, PA, USA)

6.1.3 Strains and cell culture

Dictyostelium discoideum wild-type Ax2 and DH1 cells were grown axenically in HL-5 medium (Damer and O'Halloran, 2000) supplemented with 0.6% penicillin-streptomycin (GIBCO BRL, Gaithersburg, MD) at 20^0C on Petri dishes. Clathrin heavy chain mutants were derived from Ax2 wild-type cells and clathrin light chain mutants were derived from the wild-type axenic strain NC4A2 ((Niswonger and O'Halloran, 1997a; Wang *et al.*, 2003). Both mutant strains were grown in HL-5 media supplemented with 5 μg/ml blasticidin (Calbiochem, EMD Biosciences, Inc. La Jolla, CA).

Null cells expressing GFP-AP180 (Stavrou and O'Halloran, 2006), GFP-clc (Wang *et al.*, 2003), Dajumin-GFP (Gabriel *et al.*, 1999) or GFP-Drainin (Becker *et al.*, 1999) were maintained in HL-5 nutrient media supplemented with 0.6% penicillin-streptomycin, 5 μg/ml blasticidin and 10μg/ml G418 (geniticin; GIBCO BRL, Grand Island, NY) whereas wild-type cells expressing these plasmids, were cultured in HL-5 with 0.6% penicillin-streptomycin and 10μg/ml G418. These plasmids were introduced in *Dictyostelium* cells through electroporation.

6.1.4 Disruption of *clm* by gene replacement

A 1.3 kb fragment from the 5'coding sequence of *clmA* was into the pSP72-Bsr vector (Wang *et al.*, 2002), a derivative of pBluescriptII that encodes a 1.4 kb gene for blasticidin resistance, using the BamHI and XbaI sites. Similarly, a 1.6 kb fragment from the 3' coding sequence of *clmA* was cloned into the pSP72-Bsr vector using the HindIII and XhoI sites. The two *clmA* fragments flanking the blasticidin (Bsr) resistant gene

cassette had 20 nucleotides missing from the *clmA* coding sequence which were replaced by the Bsr gene. The resulting vector, pSP72-Bsr-AP180 was linearized with BamHI and XhoI and transformed into wild-type Ax2 cells via electroporation. Transformed cells were diluted in HL-5 media supplemented with 5μg/ml blasticidin and plated in 96-well plates. Resulting clones were screened for the absence of *clmA* gene by PCR and verified for the absence of the dAP180 protein by western blot analysis.

6.1.5 Generation of adpA/AP180 double knock out cells by homologous recombination

In order to disrupt both *adpA* (gene encoding the alpha subunit of AP-2) and *clmA* (the gene encoding AP180) (Stavrou and O'Halloran T, 2006) in *Dictyostelium* cells, we first generate a single *adpA* null cell line in DH1 wild-type cells. To do that, Yujia Wen subcloned the 5'(~1.08kb) and 3' (~1.10kb) flanking regions from pSP72-Bsr- adpA construct which we used to disrupt the *adpA* gene in Ax2 wild-type cells (Wen and O'Halloran, unpublished) into pSP72-pyr plasmid using HindIII/XhoI and EcoRI sites respectively. pSP-72-pyr plasmid is a derivative of pSP72BSR vector and has the blasticidin gene replaced by a ~1.5Kb *pyr* (pyrimidine biosynthetic) gene from the pRHI30 vector. The resulting pSP72-pyr- adpA was linearized with XhoI and BglII and introduced into wild-type DH1 cells by electroporation as described previously (Wang *et al.*, 2003). Each transformation reaction was diluted into FM minimal medium (Formedium LTD. Norwich England NR13, 4HY) and plated into six 96-well plates. Resulting clones were expanded and were assessed for the disruption of *adpA* by western blot analysis using an antibody against AdpA.

We then deleted *clmA* gene in the *adpA* null cells. To do so pSP72-Bsr- AP180 (Stavrou and O'Halloran, 2006) was linearized with a BamHI and XhoI digestion and transformed in the *adpA* nulls cells via electroporation. Transformed cells were diluted in

FM minimal medium supplemented with 5μg/ml blasticidin (Bsr) and plated in 96-well plates. Resulting clones were screened for the absence of both *clmA* gene and *adpA* gene by western blot analysis.

Using the same pSP72-Bsr- AP180 construct, we also disrupted *clmA* alone in DH1 wild-type cells by homologous recombination using electroporation. Transformed cells were selected in HL-5 media supplemented with 5μg/ml blasticidin and verified for the absence of the AP180 protein by western blot as described before.

6.1.6 Construction of truncated AP180 mutants

For the construction of GFP-AP180$_{1\text{-}147}$, the full length *clmA* cloned into the pCR2.1 (TA vector) was used as a template. A primer with a BamHI restriction site was used to amplify the 5' end (TO248-GGATCCATGTCGACACCATG GGGAAAAGC) and a primer with an XhoI site was used to amplify the 3' end (TO346-CTCGAGGAATAGTTTAAGAACAGAGTAGAGAGATTTACG) to produce a 0.45 kb fragment (with no introns since the N-terminus was cloned from the cDNA library). The insert was excised from the Pcr2.1 plasmid using BamHI and XhoI restriction enzymes and ligated into pTXGFP linearized with BamHI and XhoI.

For the construction of GFP-AP180$_{239\text{-}695}$ a 5' primer with a BamHI site (TO268-GGATCCGCAATCATTGAGTTTTTCAGTTCTTCTCG) was used to amplify the full length *clmA* from the pCR2.1 vector and a 3' primer with a SmaI and an XhoI site (TO259-CCCGGGCTCGAGATAAATTTTCATTTATAAAACTTGGAAAACAACAA C). The insert was excised from the pCR2.1 vector using BamHI and XhoI (the SmaI site was lost during this step) and ligated into linearized pTXGFP using the BamHI and XhoI sites. The resulting plasmids were transformed in *Dictyostelium* wild-type, AP180 null (5H11), clathrin heavy chain null (5E2) and clathrin light chain null (2A1) cells via electroporation.

6.1.7 Site directed mutagenesis of AP180

Point mutations in the *clmA* gene (AP180) were introduced using the QuickChange Multi site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The full length *clmA* in pCR2.1 was used as a template. First the DFP motif (AP-2 binding motif) was mutated to APA using primer TO391 (DPF lower – TTTGTTACATGGGCTGGAG CTAATTCAAATGATGGTTGTGATGTAGTTGG). The NPF motif (EH-domain binding motif) was mutated to APA using primer TO394 (NPF upper – CCATCAAATT ATCATGCCCCAGCCGATCCAGCATATAATGAAAAAGC). Using primers TO394, TO392 (DPF upper – CATCATTTGAATTACGTCCAGCCCATGTAACAAATATAT CTCA and TO395 (Clathrin box upper – TAAATCATCAGCAAAATTAGCTGCTTCA TCATTTTTCTTATTA) together the DPF motif was mutated to DPA in one construct, the NPF was mutated to APA and the clathrin box LINFD was mutated to LINFA in a second construct and the DPF and NPF were mutated to APA in a third construct. The resulting constructs were excised from pCR2.1 using BamHI and XhoI and ligated into pTXGFP linearized with BamHI and XhoI. The resulting constructs were sequenced for the correct reading frame at the junction of the *gfp* gene and the mutated *clmA* gene using primer AO293. These constructs were transformed in wild-type Ax2 and AP180 null cells (5H11) using electroporation.

6.1.8 Western blot analysis.

Samples for western blotting were prepared by resuspending cells in hot sample buffer and running 1 x 10^6 cells/lane on a 10% SDS polyacrylamide gel. The gel was transferred onto a nitrocellulose membrane (0.2 micron, BioRad, Hercules, CA) and probed with a 1:2000 dilution of rabbit polyclonal antibody followed by a goat anti-rabbit

secondary antibody conjugated to horseradish peroxidase. Signal was detected using ECL kit (Piece Biotechnology, Rockford, IL, USA). When more than one primary antibody was used, the membrane was washed three times with TBS (150mM NaCl, 50mM Tris, pH7.5) before probing with another antibody.

To compare the clathrin expression level in wild-type, -adaptin null, AP180 null and AP2/AP180 double null cell lines, ~16x10^6 cells from each cell line were first collected and washed once with PDF (2 mM KCl, 1.1 mM K_2HPO4, 1.32 mM KH_2PO4, 0.1 mM $CaCl_2$, 0.25 mM MgSO4, pH 6.7). Then ~4x10^6 cells were taken from the total samples and lysed with 0.2%TritonX-100 at room temperature for 10 minutes. The protein concentration of all cell samples was measured using BioRad protein Assay (BioRad, Hercules, CA). The remaining unlysed (~12x$10^{6)}$ cells of each cell line were spun down and resuspended in hot sample buffer to 20mg/ml according to the protein sample concentration. Using those samples the protein levels of clathrin were detected by western blot using anti-clathrin heavy chain polyclonal antibody. As a loading control anti-aurora antibody was used (gift of Li and DeLozanne)

6.1.9 Endocytosis assay and differential fractionation

For the fluid-phase uptake assay, 2 mg/ml of FITC-Dextran (m.w 70kDa, Sigma-Aldrich, St. Louis, MO) was added to 3 x 10^6 cells/ml growing in HL-5 suspension cultures. Sodium azide (0.02%) was added to a control flask. To stop uptake of FITC-Dextran cells were chilled on ice. Samples were taken at 0, 15, 30, 60, 90 and 120 minute time points and centrifuged at 1100 rpm at 4^0C for 5 minutes. Cells were washed twice and resuspended in HL-5 containing 0.02% sodium azide and kept on ice until all samples were collected. All samples were centrifuged at 1100 rpm at 4^0C for 5 minutes and the pellet was resuspended in cold Na_2HPO_4 buffer. The cells were lysed with 20% Triton X-100 and fluorescence uptake was analyzed immediately using a BIORAD

VersaFluor fluorometer. A sample of the lysate was taken after the addition of Triton X-100 and assessed for protein concentration using BioRad protein Assay (BioRad, Hercules, CA).

Differential centrifugation experiments were performed according to Wang *et al,* 2003. Briefly, cells were collected and washed in isolation buffer [10mM MES (pH 6.5), 50mM $KC_2H_3O_2$ (pH 6.5), 0.5mM $MgCl_2$, 1mM EGTA, 1mM DTT and 0.02% NaN_3) with 1% protease inhibitors (Fungal Protease Inhibitor cocktail, Sigma-Aldrich, St. Louis, MO) and then lysed through a 0.5 micron polycarbonate membrane (GE Osmonics, Trevose, PA) fitted in a Gelman Luer-Lock-style filter (Gelman Sciences, Ann Arbor, MI). The cell lysates were then centrifuged at 3000 x g for 10 minutes at 4^{o}C and the resulting post-nuclear supernatant (PNS) was subjected to 100 000 x g ultracentrifugation for 60 minutes at 4^{o}C to produce a high speed supernatant (HSS) and a high speed pellet (HSP) (Wang *et al.*, 2003).

6.1.10 Fluorescence microscopy and immunostaining

Cells expressing GFP-AP180 (2 x 10^6 cells/ml) were allowed to attach on coverslips for 15 minutes at room temperature and washed briefly with PDF buffer and then overlaid with thin layer of 2% agar NA (Amersham Biosciences, Uppsala, Sweden)(Fukui *et al.*, 1987). For imaging the contractile vacuole, the agar layers were incubated in water. Cells were then fixed in 1% formaldehyde in methanol for 5 min at -20^{0}C followed by two washes with phosphate-buffered saline (PBS), rinsed briefly with distilled water and mounted on microscope slides with mounting media (MOWIOL, Calbiochem, EMD Biosciences, Inc. La Jolla, CA). The slides were allowed to dry overnight in the dark and analyzed the following day. For imaging clathrin on the contractile vacuole, we filmed live wildtype and mutant cells expressing clathrin light chain tagged with GFP in water.

For immunostaining and colocalization studies, clathrin light chain antibody (Wang *et al.*, 2003) and -adaptin antibody were prepared for immunofluorescence microscopy by pre-absorption as follows. Clathrin light chain (2A1) or -adaptin (6A5) mutant cells were grown to a density of $2x10^8$ cells/ml, centrifuged at 1500 rpm for 5 minutes and the cell pellet was resuspended in 2% formaldehyde in PBS. The cell suspension was incubated for 5 minutes at room temperature and then centrifuged at 2000 rpm for 5 minutes. The cell pellet was resuspended in 1% formaldehyde in methanol, incubated at -20^0C for 5 minutes and then centrifuged at 2000 rpm for 5 minutes. The cell pellet was resuspended in 1.5 ml of 3% Bovine Serum Albumin (Fisher Scientific, Fair Lawn, NJ) in PBS with 0.02% NaN_3. Anti-clathrin light chain or -adaptin antibody serum was added to prepared cells at a 1:5 and 1:500 dilution respectively and incubated at 4^0C overnight. The antibody-cell suspension was centrifuged for 10 minutes at 2000rpm and the supernatant was added to a new aliquot of clathrin light chain or -adaptin mutant cells and incubated at 4^0C overnight for another round of pre-absorption. This was repeated at least five times to ensure efficient absorption of non-specific antibodies from the antibody serum. Pre-absorbed antibody was added to cells that had been fixed in 1% formaldehyde in 100% methanol at -20^0C for 5 minutes and blocked with 3% BSA in PBS at 37^0C for 15minute. Fixed cells were incubated with pre-absorbed primary antibody for 1 hour at 37^0C in the dark. Cells were washed four times with PBS and incubated with Texas Red-conjugated goat anti-rabbit IgG antibody (30 μg/ml; Molecular Probes, Eugene, OR) for 1 hour at 37^0C in the dark. Cell were washed four times with PBS, rinsed briefly in water, and mounted on microscope slides as described above. For confocal microscopy we used Alexa 633 conjugated goat anti-rabbit IgG antibody (30μg/ml; Molecular Probes, Eugene, OR) instead of the Texas Red tagged secondary antibody.

To stain the actin cytoskeleton, wildtype Ax2 and AP180 null cells were allowed to attach to coverslips for 10 minutes at room temperature and then fixed in 3.7% formaldehyde in PBS for 20 minutes at room temperature followed by permeabilization with 0.2% Triton X-100 in PBS for 5 minutes at room temperature. The cells were then incubated with Texas Red phalloidin (1 unit/ml; Molecular Probes, Eugene, OR) in PBS for 20 minutes at room temperature. The cells were then washed twice with PBS and mounted on slides as described above.

Living wild-type and AP180 null cells expressing GFP-Drainin in hypotonic media were imaged using DIC and immunofluorescence microscopy. Images from DIC and immunofluorescence taken at the same time were compiled into quick time movies. Wild-type and AP180 null cells expressing GFP-V-H^+ATPase were fixed as described above without agar overlay. AP180 null and AP-1 μ-knockout cells expressing GFP-AP180 were flattened and fixed as described above.

6.1.11 Characterization of lysosomal compartments in *Dictyostelium*

To examine the size and number of lysosomes and their time of formation in wild-type cells and AP180 null cells, I used a pH sensor called Lysosensor Green DND-189 (Molecular Probes, Eugene, OR). This probe selectively localizes to acidic compartments such as lysosomes and protonation results in a fluorescent signal and detection of lysosomes. Wild-type and AP180 null cells (~ $1x10^6$ cells/ml) were allowed to attach in two-well coverslip chambers (Nulge-Nunc Int., Naperville, IL) for 15 minutes at 18^0C. The media was aspirated and 1mg/ml of LysoSensor diluted in low-fluorescence medium was added to the cells. The cells were visualized after 5 minutes using fluorescence microscopy and filmed at different time points.

6.1.12 Microscopy and confocal imaging

Cells were visualized using differential interference contrast microscopy (DIC) and fluorescence microscopy on a NIKON Eclipse TE 200 microscope. GFP and Texas Red filters were used. Images were acquired on a Photometrics cooled CCD camera, processed using Metamorph 5.0 software (Universal Imaging Co. Downington, PA USA). For imaging contractile vacuole dynamics under DIC, cells were allowed to attach in microscopy chambers in nutrient media. These were imaged under low light conditions as to not disturb the normal activity of the contractile vacuole. Another set of cells were allowed to attach in chambers first in nutrient media and then in distilled water to provide a hypotonic environment. Images of living cells were captured every 3 seconds for about 2 minutes and 27 seconds (50 frames total) and compiled into quick time movies using Metamorph software played at 6 frames per second. Still images from timelapse microscopy were used to measure the diameter of fully expanded, round contractile vacuoles. The maximum diameter size of contractile vacuoles was measured using the 100x calibrated distance tool in Metamorph software. In media, the diameter of 9, 10, 28 and 24 contractile vacuoles was measured in wild-type, AP-2 null, AP180 null and AP-2/AP180 double null cells respectively. In water, the diameter of 11, 40, 61 and 56 contractile vacuoles was measured in wild-type, AP-2 null, AP180 null and AP-2/AP180 double null cells respectively. Cells expressing Dajumin-GFP and GFP-clc were imaged in a similar way except that low fluorescence media (http:dictybase.org/techniques/media/lowflo_medium.html) was used instead of nutrient HL-5 media. For the quantification of AP180 association with the contractile vacuole, wild-type cells and clathrin light chain mutant cells expressing GFP-AP180 were analyzed. Cells were fixed and flattened as described above and imaged under DIC and fluorescence optics. Contractile vacuoles were identified using the DIC images and the presence of GFP-

AP180 outlining most of the contractile vacuole was scored using fluorescent images. For the quantification of clathrin light chain on the contractile vacuole, wild-type and mutant cells expressing GFP-clc were imaged live in water using DIC and fluorescence microscopy. Images were taken ever 3 seconds for about 150 seconds. The presence of clathrin punctae at any stage of the contractile vacuole cycle was scored.

Images of fruiting bodies during development were captured on a Zeiss Semi SR microscope with a 2.0x objective and using NIH image software. Confocal Z-series images (0.4 micron sections) of Ax2 cells expressing GFP-AP180 were obtained from Leica scanning laser confocal microscope (TCS-SP2) and processed using Leica software.

6.1.13 Membrane associated clathrin quantification using confocal imaging

Membrane associated clathrin was quantified using a Leica scanning laser confocal microscope (TCS-SP2) and processed using Leica software. Cell membranes images of were taken under the Alexa 633 filter by only focusing on the top of the cells. Differential Interference Contrast (DIC) images were also used to make sure we were focusing on the top of the cell. Clathrin light chain null cells were used as a control for non-specific binding of the antibody and all images were scaled the same. To quantify the membrane association of clathrin we collected images from 100 cells for each cell line. We used the Leica light software to circle the fluorescent area of each cell (outlining the plasma membrane) and then calculate the sum of fluorescence intensity of clathrin punctae on the cell surface. We then calculated the intensity over the area for each cell using Microsoft Excel and obtained an average for all the cells analyzed. The mean intensity/area value for each cell line was normalized against the background value of clathrin light chain cells. The difference in intensity/area between wild-type DH1, *adpA*-

cells, *clmA*- cells and *adpA*/*clamA* double null cells was compared and standard errors between every cell within the same cell line were used as error bars.

6.1.14 Quantification of AP-2 cytoplasmic punctae

DH1 wild-type, AP180 null cells and -adaptin null cells were gently flattened using agar overlay and stained with an antibody against -adaptin following fixation as described above. Images obtained from wild-type and AP180 null cells were scaled according to the background signal obtained from the stained -adaptin null images. 8-bit copies of all the images were transferred in Photoshop where the total number of punctae was measured in all cells (43 DH1 cells and 1214 punctae measured and 61 AP180 null cells 2012 punctae measured). Then the number of cytoplasmic punctae was measured in wild-type and AP180 null cells and expressed as a percentage of the total number of punctae present.

6.1.15 Development and spore morphology assay

To examine the development of *Dictyostelium* cells following nutrient depletion, $1x10^8$ wild-type (Ax2) and AP180 null cells were harvested at late log phase. Cells were washed once with starvation buffer (20mM MES, 0.2Mm $CaCl_2$, 2mM $MgSO_4$) and resuspended to $2x10^7$cells/ml in starvation buffer. 9ml of the cells suspension were plated on agar plates (1% Agar Noble [Difco Laboratories, Inc.] in starvation buffer and 6µl/ml Pennicillin/Streptomycin) and the cells were allowed to attach for 30 minutes at 18°C. Excess liquid was aspirated and plates were allowed to dry for another 30 minutes at 18°C. The cells were then incubated in the dark for about 24 hours before imaging the fruiting bodies.

To examine the morphology of *Dictyostelium* spores following starvation, wild-type and AP180 null cells were harvested from confluent plates by centrifugation at

1500rpm for 5 minutes at room temperature. The cells were washed with PDF supplemented with 0.2mM $CaCl_2$ and 2mM $MgSO_4$ and centrifuged at 1500rpm for 5 minutes at room temperature. The cells were resuspended in 10ml of PDF and pipetted on starvation plates [20mM MES pH 6.8, 0.2mM $CaCl_2$, 2mM $MgSO_4$ and 1% Noble Agar (Difco, Becton, Dickinson and company, NJ, USA)]. The cells were allowed to settle and attach for 15 minutes at room temperature and the excess PDF was aspirated. The inoculated plates were left uncovered at 18°C for 30 minutes to dry. The lids were replaced and the plates were left at 18°C in the dark until fruiting bodies developed. To harvest spores, 4-5 fruiting bodies at a time were picked using sterile toothpicks. This caused the fruiting bodies to break open and the spores to attach on the tip of the toothpick. The spores were released by swirling the toothpick in an eppendorf tube containing 500µl of PDF. 25µl of 20% Triton X-100 was added to the spores and briefly vortexed. This step lyses all the vegetative cells leaving the spores intact. The spores were then centrifuged at 10000rpm for 30 seconds, washed with PDF and centrifuged again at the same speed and time. The pellet was resuspended in 500µl of HL-5 media and transferred to a culture chamber containing 1ml of HL-5 media supplemented with Penicillin/Streptomycin (P/S). The spores were visualized using DIC microscopy.

6.2 PLASMIDS AND CELL LINES

Table 6.1: Plasmids and cells lines used in this study.

Plasmids	Description
pGEX-2T-AP180	Full length AP180 cloned in bacterial expression vector for protein purification and antibody production

pTX-GFP-AP180	Full length AP180 cloned in pTX vector, N-terminal GFP tag, G418 resistance
pSP72-Bsr-AP180	1.3kb 5' fragment of AP180 and 1.6kb 3' fragment of AP180 flanking the blasticidin resistance gene cassette to replace *clmA* gene
GFP-CLC	*Dictyostelium* Clathrin light chain, N-terminal GFP tag, G418 resistance (Wang *et al.*, 2003)
GFP-Drainin	Full length *Dictyostelium* drainin, N-terminal GFP tag, G418 resistance (Becker *et al.*, 1999)
Dajumin-GFP	Full length Dajumin cloned into pDEX RH, C-terminal GFP tag, G418 resistance (Gabriel *et al.*, 1999)
VatM-GFP	Large subunit of *Dictyostelium* V-H^+ATPase proton pump, cloned into pDXA-3H, C-terminal GFP tag, G418 resistance (Clarke *et al.*, 2002)
pTX-GFP-AP180$_{1-147}$	First 147 amino acids of *Dictyostelium* AP180 , N-terminal GFP tag, G418 resistance
pTX-GFP-AP180$_{239-695}$	Last 457 amino acids of *Dictyostelium* AP180 , N-terminal GFP tag, G418

	resistance
pTX-GFP-AP180$^{DPF/APA}$	Full length *Dictyostelium* AP180 with putative AP-2 binding motif DFP at a.a.407 mutated to APA, N-terminal GFP tag, G418 resistance
pTX-GFP-AP180$^{NPF/APA}$	Full length *Dictyostelium* AP180 with putative EH-domain binding motif NFP at a.a.459 mutated to APA, N-terminal GFP tag, G418 resistance
pTX-GFP-AP180$^{D/N/APA}$	Full length *Dictyostelium* AP180 with DFP and NPF motifs mutated to APA, N-terminal GFP tag, G418 resistance
pSP72-pyr- adpA (by Yujia Wen)	5'(~1.08kb) and 3' (~1.10kb) UTR from the AP-2 gene cloned into pSP72-pyr vector

Cell Lines	**Description**
Ax2	Wild-type axenic strain, grows in HL-5 supplemented with 0.6% P/S
DH1	Derived from Ax3 wild-type axenic strain, with the pyr5-6 gene deleted, uracil oxotroph, grows in HL-5 and FM media
AP180 null (5H11)	Derived from Ax2 parent strain, AP180 gene disrupted by the blasticidin gene cassette, grows in HL-5 supplemented with

	5µg/ml Blasticidin
AP180 null (4B8)	Derived from DH1 parent strain, AP180 gene disrupted by the blasticidin gene cassette, grows in HL-5 of FM media supplemented with 5µg/ml Blasticidin
AP-2 null (3E1) (Yujia Wen)	Derived from DH1 parent strain, -subunit of AP-2 disrupted using pSP72-pyr- adpA plasmid (by Yujia Wen)
AP-2 /AP180 null (2H5)	AP-2 and AP180 genes deleted in the DH1 parent strain using pSP72-pyr- adpA and pSP72-Bsr-AP180 pasmids, growth in FM media supplemented with 5µg/ml Blasticidin
CHC null	Derived from Ax2 parent strain with clathrin heavy chain gene deleted (Niswonger and O'Halloran, 1997a), growth in HL-5
CLC null	Derived from NC4A2 wild-type strain with clathrin light chain gene deleted (Wang *et al.*, 2003), growth in HL-5
AP-1µ null	Derived from DH1-10, µ-subunit of AP-1 deleted using full length µ-sequence cloned into pSP72-Bsr (Lefkir *et al.*, 2003) growth in HL-5 supplemented with 5µg/ml Blasticidin

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Vita

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